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CONTENTS v

THE RELATIONSHIP OF SEROLOGIC GROUPS A, B, AND C OF LANCEFIELD TO THE TYPE OF HEMOLYSIS PRODUCED BY STREPTOCOCCI IN POURED BLOOD AGAR PLATES

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The use of blood agar in the classification of the streptococci was first described by Schottmüller (1903) and more extensively studied by Brown (1919). The latter devised a descriptive system, including hemolytic, partially hemolytic, non-hemolytic, and methemoglobin-forming strains, which is widely used in defining these organisms.

The action of hemolytic streptococci on blood agar has been frequently compared with their cultural and biochemical reactions. However, since the separation of these organisms into groups on a serologic basis by Lancefield (1933), few studies have been made of the relationship between the various serologic groups and the formation of hemolysin.

Todd (1934) and Long and Bliss (1937) have studied the formation of soluble hemolysin by the members of most of these serologic groups and have found them readily capable of forming this substance in every instance. Todd has demonstrated that an antigenic hemolysin is produced only by strains in Group A, and he expressed the opinion that the hemolysin derived from each group possessed demonstrably different reactions to physical and chemical agents. The nature of the type of hemolysis of these varying bacterial products in blood agar was not studied.

Brown (1937; 1939) has recently described the characteristic appearance in poured blood-agar plates of the members of the

serological Group B. Two zones of hemolysis, an inner clear zone and an outer zone containing unhemolyzed red blood cells, were observed under favorable conditions after 48 hours' incubation at 37°C.

It is the purpose of this paper to report the results of a study of 1179 strains of hemolytic streptococci in poured blood agar and to correlate the observed serological and hemolytic characteristics. It will be shown that the type of hemolysis produced by Group A is uniform, characteristic, and distinguishable from all other serologic groups of streptococci, that Group B also has a unique type of hemolysis, and that Group C has a uniform type of hemolysis, although some of the higher groups also produce similar hemolysis. These different types of hemolysis can be observed only when standardized poured blood-agar plates are used and not the more commonly used streak plates.

METHODS

Strains of hemolytic streptococci which had been isolated from patients with various diseases were plucked from streak blood-agar plates for more intensive study. Differentiation of the various strains was made both by means of serologic tests and by the use of pour plates.

Serologic grouping was performed by the micro-precipitin method of Brown (1938), sera for Groups A, B, and C being available.[1]

Poured plates were prepared by inoculating 12 ml. of veal-infusion agar containing 0.7 ml. of defibrinated horse blood with a drop of diluted broth culture, which was then mixed and poured in a $3\frac{1}{2}$ inch sterile petri dish. These plates were observed after 24 and 48 hours in the incubator at 37°, and after 24 hours in the icebox. This is approximately the method previously described by Brown (1919).

The appearance of the surface colonies was studied by streaking a loopful of culture on similar blood agar plates and incubating for 24 hours at 37°C.

Hemolysis was recorded as complete when the zone of clearing contained no red blood cells, partial when a large number but not all the cells had been destroyed, and slight if only minimal evidence of hemolysis was present. Plate I shows Groups A, B, and C (magnification 1½ ×). 1) After 24 h. incubation at 37.5°; 2) after 48 h. incubation; 3) after 24 h. refrigeration at 5°.

RESULTS

Group A

Of the 1179 strains studied by the method previously described, 1104 had similar characteristics. All the strains having these characteristics were found to belong to Group A according to the Lancefield classification.

Surface. On the surface of blood agar plates, the colonies were either medium-sized, pearly white, dome-shaped colonies or large, irregular, flat, translucent colonies corresponding to the M and F variants of Ward and Lyons (1935). Zones of complete hemolysis, varying from one to 2 millimeters in diameter, with sharply defined margins, surrounded the colonies of a majority of the strains studied. Around a large number of the colonies, however, there were only small zones of partial hemolysis, even though the same strains in poured plates showed the characteristic hemolysis as described below.

Poured blood-agar plates. At the end of 24 hours' incubation individual colonies were surrounded by an area of almost complete hemolysis and the borders were sharp and clean cut. The hemolytic zones varied from one and one-half to three and one-half millimeters in diameter, the average being two and one-half millimeters.

After incubating the cultures for 48 hours at 37°C., it was noted that the area of hemolysis had increased about one millimeter in diameter and the zone of hemolysis was absolutely clear. No red blood cells could be seen within it when they were examined by the microscope. The margins were very sharp and clean cut. For a given strain, all the zones of hemolysis about a colony were almost exactly the same size. There was only slight varia-

tion, approximately 2 mm., in the size of the zone of hemolysis from one strain to another.

When the plates were again examined after they had been placed in the ice box at 5°C. for 24 hours, the appearance of the colonies and zones of hemolysis showed no change.

From these observations, it seemed clear that serological group A hemolytic streptococci showed the following distinguishing characteristics in blood agar: 1) the zone of hemolysis about the colony was complete; 2) the size of the hemolyzed zone was practically constant for the individual strains; 3) the size of the hemolyzed zones varied very little in different strains compared with the variation in other groups; 4) the border of the zone of hemolysis was sharp and definite.

Group B

Twenty-three strains of Group B were isolated and their features on the surface and in poured blood-agar plates were noted. All strains which were grouped serologically as Group B possessed the following characteristics, and every strain could be placed in Lancefield's serological Group B.

Surface. The colonies on the surface of blood-agar plates were large, flat, and gray in appearance and they were surrounded by a very narrow zone of partial hemolysis. This seemed to be quite characteristic but they were occasionally confused with other serologic groups.

Poured blood agar plates. In contrast to the Group A streptococci, the zone of hemolysis 24 hours after incubation at 37°C. showed an area of incomplete hemolysis about 2½ millimeters in diameter surrounding the colony. The border of the zone of hemolysis was hazy and diffuse, and many incompletely hemolyzed red blood cells were distributed through this zone of incomplete hemolysis.

At the end of 48 hours' incubation the picture had changed somewhat, the zone of hemolysis had increased about one millimeter in size, and it was complete or nearly so. At this time, there was in addition a faint but definite band of hemolysis, about one-half to one millimeter wide, around the clear-cut

hemolyzed zone. When these plates were placed in the ice box for 24 hours, the outside zone of hemolysis had increased in both size and degree until it measured between one and 2 millimeters wide (Plate 1). Therefore, we could conclude that Group B streptococci could be distinguished from the other groups by the band of slight or partial hemolysis surrounding a zone of complete or nearly complete hemolysis. The band occurred in all strains either after 48 hours' incubation or following 24 hours' refrigeration. Brown (1937; 1939) has described the appearance of this distinctive double zoning hemolysis. We have not encountered any Group B strains which failed to show the double zone of hemolysis, although Brown has described such strains.

Group C

There were 19 proved serologic Group C streptococci in the group. They all presented the same type of hemolysis and the same appearance on surface plates, and they could be distinguished quite readily from Groups A and B organisms in poured blood-agar plates. Four more strains which, in poured agar plates, were identical with Group C could not be grouped with serums A, B, or C.

Surface. On the surface the colonies were not entirely distinctive. The zones of hemolysis were larger than those of Group A but they were less complete and the margins were more diffuse.

Poured blood agar plates. In the poured blood-agar plates, after 24 hours' incubation at 37°C., the colonies appeared large, and were surrounded by an area of clear or nearly clear hemolysis which became progressively less so as one approached the periphery. The diameter of the zone of hemolysis was 3 to 5 millimeters but the borders were indefinite so that accurate measurement was impossible. Following 48 hours of incubation, a great increase in the size of the zone of hemolysis occurred, the diameter varying between 5 and 9 millimeters in many instances. Immediately about the colony there was complete hemolysis but the periphery remained very diffuse and was indistinct. Following refrigeration, there was practically no change in the appearance of the colony. In a word, then, the

Group C streptococci could be distinguished easily from Groups A and B by the very large zone of hemolysis which was complete for 3 millimeters or more in the center and which gradually became less complete until it was only slight at the periphery.

Unidentified Strains

Nineteen strains which were isolated did not resemble either A, B, or C and, since those were the only group sera available to us, we were unable to identify them.

Groups D, E, F, G, H, and K

Relatively few strains of Groups D, E, F, G, H, and K were available for study. Those described below were sent to us through the kindness of Dr. James M. Sherman of New York and Dr. E. A. Bliss of Baltimore. Previously they had been grouped according to the serological groups of Lancefield. We wanted to see if these groups had a cultural characteristic in poured blood-agar plates which was distinctive for the group to which they belonged. We were unable to distinguish these groups by this method. Although we had only 2 or 3 strains of each group, there seemed to be no comparison between strains of a given group. However, almost every group contained a strain which was indistinguishable in appearance in blood agar from the appearance of all the Group C strains. None of the strains of Groups D, E, F, G, H, and K were confused with Groups A or B, since they failed to produce either the completely hemolyzed zone with its sharp border, which is characteristic of Group A, or the double zone of hemolysis in Group B.

COMMENT

No satisfactory explanation has been offered for the varying effects of streptococci on blood agar. Evidence is presented in this paper which suggests that at least some of the serologic groups of Lancefield are constant in their various effects on this substance. The underlying mechanisms for these differences remain obscure, except to further suggest, as emphasized by Todd (1934), that the hemolysins of the streptococci of the various groups are distinct substances.

In the 1179 strains discussed in this paper, the type of hemolysis described for the members of Group A has proved characteristic in every instance. Strains of Group B also have always produced a highly distinctive hemolytic pattern. While all strains of Group C were consistent, they could not be distinguished from several strains in the higher groups. From the relatively few strains of the other groups it would not appear that these groups have a characteristic cultural growth in blood agar.

Practically, these variations have proved of great value as checks on serological studies. On various occasions difficulties were encountered in the serologic groupings: one lot of Group A serum showed a false precipitate in all strains; Groups B and C serum required a great deal of care in obtaining the right proportion of serum and antigen and, on occasions, the antigen was not sufficiently strong. On repeated serologic tests they always checked with the blood-agar grouping. Rapid approximations as to the presence or absence of human virulent strains may also be made from material such as throat swabs and sputa if these be suitably diluted and poured in standard horse blood agar plates and examined after 24 and 48 hours' incubation at 37°C. in the absence of available serum.

Brown (1919) has emphasized, and it is important to point out, that these characteristic changes will not be demonstrable unless poured plates are used, as the appearance of colonies on the surface has very little correlation with the widely differing types of hemolysis described in this paper.

From the evidence above, it is likely that the development of hemolysis in blood agar is as constant a function of the organism in certain groups as is the development of group specific substance.

SUMMARY AND CONCLUSIONS

1. Strains of hemolytic streptococci, members of Lancefield's serologic Groups A to K, were studied in poured blood-agar plates.

2. The type of hemolysis produced by strains of Group A was constant and characteristic in every instance.

3. All members of Group B which were studied produced characteristic hemolysis with "double zoning."

4. Members of Group C produced a characteristic hemolysis

which, however, could not be differentiated from various strains in higher groups.

5. The few strains of Groups D, E, F, G, H, and K did not show a group-specific hemolytic effect in blood agar.

6. It is suggested that the appearance of streptococci in poured blood-agar plates may be used as a check on serologic tests and as a satisfactory method of approximating the group of unknown strains in the absence of grouping serum.

7. Standardized blood-agar plates must be used or the described changes will not be observed.

We wish to thank Miss Eleanor Fleming for her assistance in the serologic grouping.

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PLATE 1. HEMOLYSIS AFTER: (1) 24 H. AT 37.5°; (2) 48 H. AT 37.5°; (3) AN ADDITIONAL 24 H. AT 5°
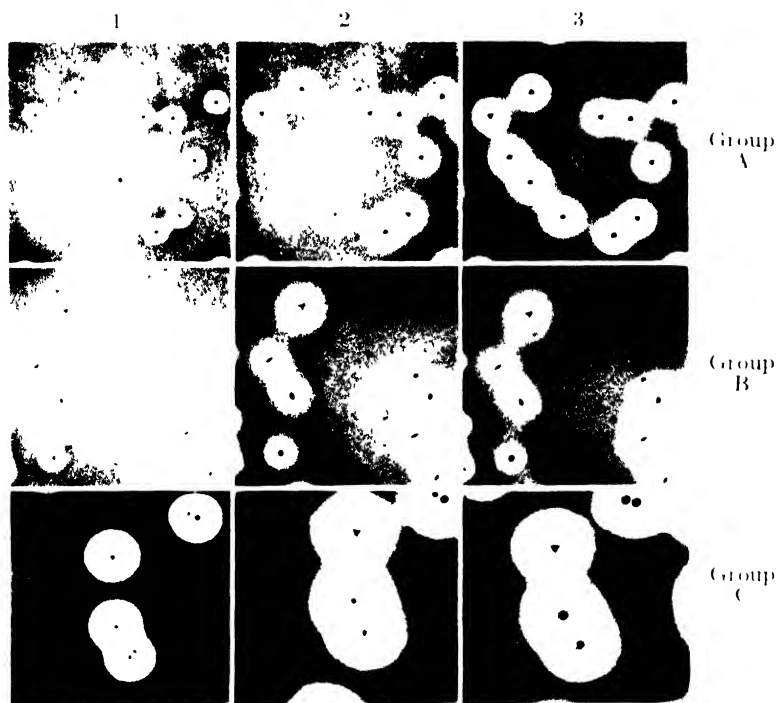
Lowell A. Rantz and Marjorie I. Jewell: Serologic Groups and Hemolysis

THE VALUE OF CERTAIN TESTS IN THE DIFFERENTIATION OF LACTOBACILLUS BULGARICUS FROM LACTOBACILLUS ACIDOPHILUS

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At the 1920 meeting of the Society of American Bacteriologists a paper, published only in abstract form (Sherman, 1921), presented some characteristics of *Lactobacillus bulgaricus*, differentiating this organism from certain other lactobacilli. Eighteen cultures of *Lactobacillus bulgaricus* were then studied; a number of *Lactobacillus casei*; a few cultures from feces, assumed to belong to the *Lactobacillus acidophilus* group; and one "authentic" culture, each, of *Lactobacillus acidophilus* and *Lactobacillus bifidus*. The supposed culture of *Lactobacillus bifidus* appeared to be entirely similar to the culture of *Lactobacillus acidophilus*. The gas-producing lactobacilli were not included in the study, nor the homofermentative *Lactobacillus delbrueckii* group.

As this work was done before the intensive studies of *Lactobacillus acidophilus*, the doubt then existing concerning the identity of this organism, and the question of whether or not it was really represented in our collection, made inadvisable the publication of the data in full. However, continued study of authentic cultures of *Lactobacillus bulgaricus*, and of *Lactobacillus acidophilus*, obtained from qualified specialists, convinced us that the tests then indicated are of value in the differentiation of these species.

There is no longer need for confusing the *Lactobacillus casei* group with the acidophilus-bulgaricus groups and other members of the lactobacilli which grow at relatively high temperatures. This paper will discuss the differentiation of *Lactobacillus bulgari-*

cus from *Lactobacillus acidophilus* by tests long used in this laboratory. No claims are made for the value of these tests if applied to other closely related species; and we recognize the possibility that the tests suggested may prove of less value as more knowledge is accumulated and the natural confines of, and variability in, these species become better established.

PREVIOUS WORK

In our earlier work, a number of points of difference were found between *Lactobacillus bulgaricus* and the intestinal cultures which were supposed to represent the acidophilus group.

The alkaline limits of growth

All of the cultures of *Lactobacillus bulgaricus* were inhibited in broths having a pH of about 7.5, while the intestinal lactobacilli grew well at pH 7.8 or above. This test was inspired by the work of Cohen and Clark (1919) on the rate of growth of bacteria at various hydrogen ion concentrations. They tested, among other bacteria, a culture of *Lactobacillus bulgaricus* and found that this culture did not initiate growth in a medium of pH 7.5. Our further tests confirmed the finding of Cohen and Clark with all of the cultures of *Lactobacillus bulgaricus* then available and brought out the additional point that other lactobacilli will grow in more alkaline media. For the past fourteen years we have used as a regular laboratory test, with large classes, the ability to grow in media of pH 7.8 as a differential test between *Lactobacillus bulgaricus* and *Lactobacillus acidophilus*. In our hands, this has proved one of the best means of distinguishing these species.

Although these findings have not been applied by others as a differential method, some observations have been made which bear upon the subject. Orcutt (1926) studied the acid and alkali tolerance for growth of cultures of *Lactobacillus acidophilus*, isolated from the digestive tract of calves, and concluded that the limit on the alkaline side appeared to be about pH 7.8 to 8.0, though some freshly isolated cultures made little or no growth at pH values of 7.7 to 7.8. On the other hand, Weiss and Rettger

(1934) concluded that *Lactobacillus acidophilus* is usually inhibited above pH 6.8. This divergent result is perhaps explained, as Professor Rettger has suggested in personal correspondence, by the fact that their experiments were conducted with washed cells as the inocula.

Inhibition by salts

It was found that *Lactobacillus bulgaricus* is more easily inhibited in artificial media by the addition of phosphates or sodium chloride. For example, *Lactobacillus bulgaricus* was inhibited in broths containing only about 2 per cent sodium chloride, while the intestinal lactobacilli were considerably more tolerant. This test, using 2.5 per cent sodium chloride, has been used during recent years, since the availability of authentic cultures of *Lactobacillus acidophilus*, with consistently good results. This method appears to offer a fairly good margin of safety for a differential test since, in our experience, *Lactobacillus bulgaricus* is usually inhibited by only 2 per cent sodium chloride whereas many strains of *Lactobacillus acidophilus* are able to grow in the presence of 5 per cent of this salt.

Ability to grow in artificial media

Although many lactobacilli grow poorly in laboratory media, it was our experience that *Lactobacillus bugaricus* was much more delicate in this respect than were the intestinal types supposedly belonging to the acidophilus group. For example, using a relatively simple broth containing lactose, peptone and yeast extract, *Lactobacillus bulgaricus* will grow well for one or two transfers but is unable to grow through successive transplants in such a medium. *Lactobacillus acidophilus* is markedly more hardy and adaptable in this respect, growing well through ten successive transfers.

Production of carbon dioxide

While neither of the species in which we are here interested belongs to the group of lactobacilli which produce significant amounts of carbon dioxide, they do of course produce minute

amounts of this gas which may be demonstrated with appropriate methods (Eldridge and Rogers, 1914). It was noted that *Lactobacillus bulgaricus* produced less carbon doxide than the lactobacilli then supposed to belong to the acidophilus group. Although we have not studied this characteristic in recent years with authentic strains of *Lactobacillus acidophilus*, this point appears to have been confirmed by the work of Curran, Rogers and Whittier (1933), with a large collection, and also with a few authentic cultures of *Lactobacillus acidophilus* and *Lactobacillus bulgaricus* by Dr. Paul Hegarty, formerly of these laboratories. As this is not a convenient test for routine purposes, we have not verified it by class work with students, nor was it used in the present study in view of the other simple tests of equal or superior integrity.

Minimum temperatures for growth

As is well known, *Lactobacillus bulgaricus* and *Lactobacillus acidophilus* both belong to the more or less heat-tolerant lactobacilli which grow at relatively high temperatures and may be sharply distinguished from *Lactobacillus casei* and its kindred varieties or species which have lower minimum and maximum temperatures of growth. In our earlier work no strain among the authentic cultures of *Lactobacillus bulgaricus* was able to grow at 15°C. whereas all of the clutures supposedly belonging to the acidophilus group grew at this temperature. Our tests in recent years of authentic strains of *Lactobacillus acidophilus* have indicated that this organism usually grows at 15°C., whereas the true *Lactobacillus bulgaricus*, in our experience, seldom does.

These points were not confirmed by Curran, Rogers and Whittier (1933) who worked with a more varied collection of cultures, and we must not assume that their strains of *Lactobacillus acidophilus* were any less "authentic" than our own. With respect to *Lactobacillus bulgaricus*, it is not possible to correlate the findings of Curran, Rogers and Whittier with our results, as their collection of organisms, other than *Lactobacillus acidophilus*, apparently contained *Lactobacillus casei* and other types, with very few if any cultures of *Lactobacillus bulgaricus*. This fact

was recognized and clearly stated by them. A number of other investigators have also reported the minimum temperature for the growth of *Lactobacillus acidophilus* as being about 20°C.

Maximum temperatures for growth

It has been our observation that *Lactobacillus acidophilus* will not grow at 50°C. and this fact has now been confirmed by Curran, Rogers and Whittier (1933) and Orla-Jensen, Orla-Jensen and Winther (1936), both sets of investigators finding that 48°C. is the upper limit for the growth of this organism. On the other hand, *Lactobacillus bulgaricus* usually grows at 50°C., and we have used this means of differentiation for many years in class work.

For reasons which are now obvious, this test has frequently failed in recent years when old stock cultures of *Lactobacillus bulgaricus* were used (Sherman and Hodge, 1936). The difference becomes more marked when freshly isolated strains of *Lactobacillus bulgaricus* are employed, many of which grow vigorously at temperatures exceeding 55°C.

CONFIRMATORY EXPERIMENTS

After the foregoing discussion of our earlier work the experimental data gathered in the present studies may be briefly presented.

Methods

For testing the ability of the organisms to make repeated growth in a relatively simple medium, a broth was used which contained 1 per cent each of lactose, peptone and yeast extract (Bacto). Loop transfers were made until the culture failed to grow; or ten transfers were completed.

Salt tolerance was tested in the same basic lactose-peptone-yeast extract broth to which 2.5 per cent sodium chloride was added.

The test based upon the alkaline limits of growth offers slight technical difficulties in that the reaction may change considerably upon sterilization with heat at such a high pH. This may of

course be obviated by adjusting the medium after sterilization or by sterilization by filtration, but the pH of unbuffered alkaline media frequently falls considerably upon standing. One of us (Hodge) has devised a medium which appears to give a constant pH of about 7.8 after sterilization in the autoclave. This medium, which was used in the experiments here reported, has the following composition:

	Per Cent
Lactose	1.0
Peptone	1.0
Yeast extract (Bacto)	1.0
Sodium citrate	0.5
Sodium bicarbonate	0.065

The pH of this broth is about 7.2 before sterilization and rises to 7.80 to 7.84 after sterilization, at which point it remains practically unchanged for one week at 37°C. All pH values were determined by use of the hydrogen electrode. Tubes of this medium were inoculated, incubated at 37°C. and observed for growth and acid production.

To determine the limiting temperatures for growth, litmus milk was used which was supplemented by the addition of 0.5 per cent peptone and 100 ml. of tomato juice per liter, adjusted to pH 6.5. Excellent growth is obtained in this medium even with freshly isolated cultures which may grow poorly in ordinary sterilized milk. This medium was inoculated with one drop of a vigorous 24–48 hours old culture; the test tubes were closed with sterile rubber stoppers to prevent evaporation and resultant cooling, and adjusted in a water bath to the desired temperature before being placed in the incubator. The 15°C. tubes were examined after two months, whereas the high-temperature cultures were observed for acid production after one week.

Cultures

In all, 152 cultures were used of what we shall designate as the "bulgaricus group." Five were of *Lactobacillus bulgaricus* obtained from authoritative sources; 142 from market milks and one from a sample of Egyptian "leben," and identified by us as

Lactobacillus bulgaricus. Also included in the "bulgaricus group" were four cultures isolated from an Armenian "mazun" which appear to be identical with the "Bulgaricus Type I" of White and Avery (1910) and the lactobacillus described by Orla-Jensen (1919) as *Thermobacterium jugurt.* This organism, which we shall refer to as "*Lactobacillus jugurt*," differs from the typical *Lactobacillus bulgaricus* in a more rapid and vigorous growth in milk, the production of much more acid, the formation of inactive lactic acid, and in certain slight morphological and colonial characteristics.

As representatives of the "acidophilus group," 43 cultures were used: six stock cultures from leading specialists, 14 strains isolated from human feces, 21 cultures isolated from miscellaneous sources, and two cultures of *Lactobacillus bifidus* obtained from Professor L. F. Rettger. The cultures from "miscellaneous sources" were isolated from human saliva, the throats of cows, the fourth stomach of calves, and milk. As we hold no opinion on the disputed point of whether or not the "true" *Lactobacillus acidophilus* should be more rigidly restricted from the standpoint of source and intestinal implantability, we emphasize "acidophilus group," but it should be stated that on the basis of detailed study all of these cultures agreed with the authoritative descriptions of the species. The cultures of *Lactobacillus bifidus* were from the collection of Weiss and Rettger (1934) concerning which they concluded ". . . that *L. bifidus* should be regarded as a variant of the species in which *L. acidophilus* is the central type."

Experimental findings

The results obtained are given in table 1. It will be noted that perfect separations were obtained with the tests based upon the ability to make repeated growth in a simple medium, and on salt tolerance. Although equally good results were obtained with the test based on alkali tolerance, the margin of safety appears to be rather thin and difficulty might be occasionally experienced with delicate cultures of *Lactobacillus acidophilus.*

The tests based on the temperature limits of growth did not give perfect results but are nevertheless of value. Only rarely

does *Lactobacillus bulgaricus* grow at 15°C. whereas members of the acidophilus group usually do, under the favorable conditions used in our experiments.   At 50°C., none of the cultures of *Lactobacillus acidophilus* grew; freshly isolated cultures of *Lactobacillus bulgaricus*, on the other hand, almost always grow at this temperature, but old stock cultures frequently fail.   The loss of thermophilic properties in laboratory cultures of *Lacto-*

TABLE 1

Lactobacillus bulgaricus and Lactobacillus acidophilus

SPECIES OR VARIETY	NUMBER OF CULTURES STUDIED	GROWTH THROUGH 10 TRANSFERS IN SIMPLE MEDIUM	GROWTH IN BROTH CONTAINING 2.5 PER CENT NaCl	GROWTH IN BROTH AT pH 7.8	GROWTH AT 15°C.	GROWTH AT 50°C.
"Bulgaricus group"						
L. bulgaricus (authentic cultures)	5	—	—	—	—	3+
						2−
L. bulgaricus (from milk). . .	143	—	—	—	136−	135+
					7+	8−
"L. jugurt" (Armenian mazun) .	4	—	—	—	—	+
"Acidophilus group"						
L. acidophilus (authentic cultures).	6	+	+	+*	4+	−
					2−	
L. acidophilus (human feces)	14	+	+	+	11+	−
					3−	
L. acidophilus (miscellaneous sources)....	21	+	+	+	+	−
L. bifidus (Professor L. F. Rettger).	2	+	+	+	+	−

* Two cultures of *L. acidophilus* grew weakly in broth at pH 7.8.

bacillus bulgaricus has been previously noted (Sherman and Hodge, 1936).

CORRELATION WITH OTHER TESTS

As the purpose of this paper is to present the results obtained with certain neglected tests which have long been in use in this laboratory, a detailed consideration of the characteristics of the species involved is not included.   It is essential, however, to

show how these tests agree with the other differential methods in use.

Beijerinck (1901), probably the first to study the true *Lactobacillus bulgaricus*, noted a complete absence of maltose fermentation in the levo-lactic-acid-producing variety which he designated as *Lactobacillus longus*. Rahe (1914, 1918) emphasized maltose and sucrose, finding that *Lactobacillus acidophilus* fermented these sugars, whereas *Lactobacillus bulgaricus* did not ferment maltose and usually did not ferment sucrose. Kulp and Rettger (1924) noted that *Lactobacillus bulgaricus* cannot ferment *unheated* fructose whereas *Lactobacillus acidophilus* does. In a general way, these results have been confirmed by a majority of the workers on this problem, though there have been many vigorous dissenters; some recent investigators ascribing no value to fructose, maltose and sucrose for the differentiation of these species. These discordant results, obtained in many cases with the same stock cultures, are explained to some extent by Weaver (1932) who has shown that strains of *Lactobacillus bulgaricus* which fail to act on fructose, maltose or sucrose may ferment these sugars when the tests are performed with a more favorable basic medium. Hodge (1937) has shown, further, that *Lactobacillus bulgaricus* may sometimes acquire the ability to ferment maltose and sucrose after long laboratory culture.

In our own work, using a basic medium containing 1 per cent each of yeast extract and peptone, none of the cultures of *Lactobacillus bulgaricus* fermented unheated fructose or maltose when freshly isolated, although the ability to ferment maltose is possessed by some old stock strains and was acquired by some of our isolations after laboratory culture for two years in milk. Only a small minority of strains of *Lactobacillus bulgaricus* ferment sucrose when first isolated, but this property is less rare among old laboratory cultures. In keeping with the findings of others, our cultures of *Lactobacillus acidophilus* ferment fructose, maltose and sucrose.

We now come to the much-maligned sodium ricinoleate test of Albus and Holm (1926). Kopeloff, a leading authority on these groups of organisms, has reported good results with the

method in his laboratory (Kopeloff and Beerman, 1927, Kopeloff and Kopeloff, 1937), but the test has been severely criticized by several workers. Some of the criticisms may be sound on academic grounds, but as a practical test we are not concerned as to whether the results obtained with it are due to the surface tension *per se* or only to the inhibitory action of the depressant used.

In a sodium ricinoleate broth with a surface tension of 37.5 dynes at 37°C., not one of our 152 cultures representing the "bulgaricus group" was able to grow. All of the cultures of the "acidophilus group" grew in this medium, though it should be mentioned that the same two "authentic," but atypical, cultures which failed to grow at 15°C., and grew weakly at pH 7.8, also grew weakly in the sodium ricinoleate medium.

Kulp (1929) has shown that phenol or indole may be incorporated in media in such concentrations that *Lactobacillus bulgaricus* is inhibited while *Lactobacillus acidophilus* is able to grow. We have used the phenol tolerance test of Kulp with good, though not perfect, results. With a favorable agar medium containing a phenol concentration of 1 to 300, only four of the 43 cultures representing the acidophilus group failed to grow; although a substantial proportion of the strains of *Lactobacillus bulgaricus* grew, the vast majority of them were inhibited.

NOTE

In the beginning of this paper it was stated that no claims are made for the tests here described if applied to other closely related species, a warning of equal pertinence in connection with the other differential methods which have been used for this purpose. With the exception of the maximum temperature of growth, all of the methods which have been used by us and others, for this purpose, are tests for the ability of *Lactobacillus acidophilus* to do things which *Lactobacillus bulgaricus* cannot do. On the basis of present knowledge, it appears that the inability to grow in the presence of various mildly inhibitory factors, the inability to make repeated growth in a relatively simple medium, and the failure to ferment certain sugars, are characteristics peculiar to *Lactobacillus bulgaricus* and a few closely related varieties.

On the other hand, although the reactions obtained with these tests are characteristic of *Lactobacillus acidophilus* they are by no means peculiar to it, being shared, in fact, by a majority of the known lactobacilli.   For example, *Lactobacillus casei*, with these tests, reacts as does *Lactobacillus acidophilus*, the differentiation of these species resting on quite different bases.   More confusing is the type which has been described by Orla-Jensen (1919) under the name of *Thermobacterium lactis*.   This organism reacts more as does *Lactobacillus acidophilus* on these tests, but resembles *Lactobacillus bulgaricus* in having a substantially higher maximum temperature of growth and in the production of levo lactic acid.

With the exception of a few groups, so little is now known of the lactobacilli that the stage of "new species," "variant types" and "intermediates" has scarcely yet been entered.

SUMMARY

Lactobacillus bulgaricus is unable to make repeated growth in a lactose-peptone-yeast extract broth, is unable to grow in media containing 2.5 per cent sodium chloride, and can not grow in broth with a reaction of pH 7.8.   *Lactobacillus acidophilus*, on the other hand, is not inhibited in such mildly alkaline or saline media, and grows well through ten successive transfers in the relatively simple broth.

Lactobacillus bulgaricus very rarely grows at 15°C. whereas *Lactobacillus acidophilus* usually grows at this temperature. At 50°C., *Lactobacillus acidophilus* apparently never grows; newly isolated strains of *Lactobacillus bulgaricus* nearly always grow, though old laboratory cultures frequently fail.

These tests have been found to correlate with other differential methods.

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CATION ADSORPTION BY BACTERIA[1]

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At the present time there is a great deal of interest in the mechanism of microbial nutrition. Any knowledge of the manner in which the bacterial cell functions as a physico-chemical unit should assist in elucidating problems of nutrition.

It is a well-known fact that proteins (Docking and Heymann, 1939) as well as inorganic colloids (Jenny, 1932) possess an adsorption capacity for cations. Since the bacterial cell contains proteins, approaches inorganic colloids in size, and is negatively charged (Tittsler, 1938; Kendall, 1925), it seems logical that it might function as an agent in the adsorption of positively charged ions.

Robbins (1926) showed that plants such as *Elodea* and potato tuber would adsorb cations and anions. Stearn and Stearn (1928) indicated from their studies of simple proteins that bacteria might adsorb cations and anions. Jenny and Overstreet (1939) stated that barley roots exhibit a positive cation adsorption capacity of 8 m.e. per 100 grams of plant material. As far as the writer is aware, no one has undertaken to demonstrate such a phenomenon quantitatively for bacteria.

An understanding of the mechanism by which a bacterial cell takes ions from its environment and concentrates them in a limited area around and, possibly, inside the cell, with an electrical force sufficient to limit their freedom of movement, might help to elucidate some of the laws governing the physiological activity of these organisms. For example, knowledge relative to the degree of adsorption of a mineral nutrient by a bacterial cell might help to explain the role of such an element in growth. Only by carefully controlled physico-chemical studies can the

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[1] Contribution No. 185 from the Department of Bacteriology.

function and interrelation of the various cations involved in bacterial nutrition, growth, and disinfection be determined.

Since the bacterial cell is known to carry a negative charge and is also known to form acids (carbon dioxide and water) the following equations will serve to indicate possible cation adsorption by bacteria.

$$(\text{Bacterial cell}^{n-}) + nH^+ \rightleftarrows (\text{Bacterial cell}^{n-})\,(nH^+)$$

$$(\text{Bacterial cell}^{n-})\,(nH^+) + \begin{array}{l}(nNaOH)\\(nKOH)\\(\tfrac{1}{2}\,nCa(OH)_2)\\(\tfrac{1}{2}\,nMg(OH)_2)\end{array} \rightleftarrows$$

$$(\text{Bacterial cell}^{n-})\begin{array}{l}(nNa+)\\(nK^+)\\(\tfrac{1}{2}\,nCa^{++})\\(\tfrac{1}{2}\,nMg^{++})\end{array} + nHOH$$
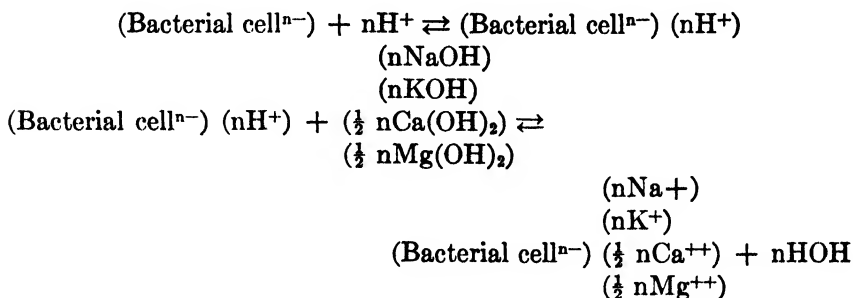
From the above equations it might be expected that a bacterium growing in a balanced medium would adsorb Na^+, K^+, Ca^{++}, Mg^{++}, etc., from the medium. If, then, an excess of H ions were added to these cells as HCl, it might be expected that the Na^+, K^+, Ca^{++}, Mg^{++}, etc., would be replaced by the H^+. The basic ions released from the adsorption complex would unite with the Cl^- ions to form the respective water-soluble chlorides which could be removed by washing as indicated in the following equation.

$$(\text{Bacterial cell}^{n-})\begin{array}{l}(nNa^+)\\(nK^+)\\(\tfrac{1}{2}\,nMg^{++})\\(\tfrac{1}{2}\,nCa^{++})\\(\text{etc.})\end{array} + nHCl \rightleftarrows$$

$$(\text{Bacterial cell}^{n-})\,(nH^+) + \begin{array}{l}(nNaCl)\\(nKCl)\\(\tfrac{1}{2}\,nMgCl_2)\\(\tfrac{1}{2}\,nCaCl_2)\\(\text{etc.})\end{array}$$
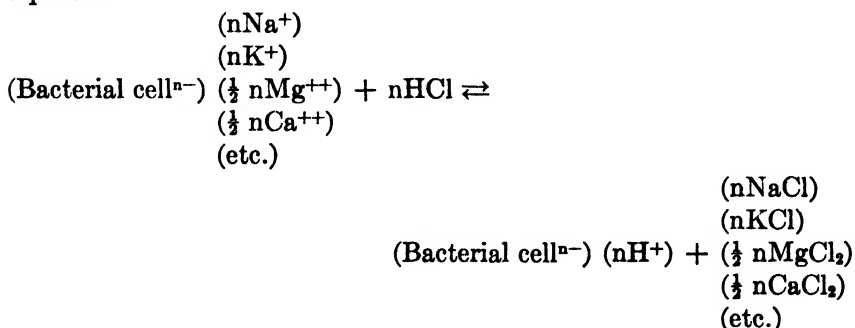
The H-bacteria thus formed should be titratable with $Ca(OH)_2$ and the amount of $Ca(OH)_2$ used should be a measure of the H^+ adsorbed, or the cation adsorption capacity of the bacterial cells.

$(\text{Bacterial cell}^{n-})\ (n\text{H}^+) + \frac{1}{2}\ n\text{Ca(OH)}_2 \rightleftharpoons$
$$(\text{Bacterial cell}^{n-})\ (\tfrac{1}{2}\ n\text{Ca}^{++}) + n\text{HOH}$$
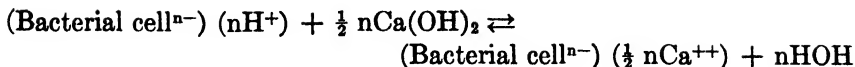
By drying an aliquot of the suspension of bacteria at 105°C. and weighing, values obtained as above could be expressed as m.e. (milligram equivalents) of cations per 100 grams of bacteria.

Theoretically it should be possible to substitute other chlorides such as methylene blue chloride, mercuric chloride, etc., for the hydrogen chloride, and since the methylene blue ion can be measured colorimetrically and the mercury ion functions as a lethal agent, they afford two additional tools for testing out the adsorption hypothesis. Such theoretical reactions are indicated in the following equations.

$$(\text{Bacterial cell}^{n-}) \begin{pmatrix} (n\text{Na}^+) \\ (n\text{K}^+) \\ (\tfrac{1}{2}\ n\text{Mg}^{++}) \\ (\tfrac{1}{2}\ n\text{Ca}^{++}) \\ (\text{etc.}) \end{pmatrix} + n\text{MB}^+\text{Cl}^{-2} \rightleftharpoons$$

$$(\text{Bacterial cell}^{n-})\ (n\text{MB}^+) + \begin{pmatrix} (n\text{NaCl}) \\ (n\text{KCl}) \\ (\tfrac{1}{2}\ n\text{MgCl}_2) \\ (\tfrac{1}{2}\ n\text{CaCl}_2) \\ (\text{etc.}) \end{pmatrix}$$

or

$$(\text{Bacterial cell}^{n-}) \begin{pmatrix} (n\text{Na}^+) \\ (n\text{K}^+) \\ (\tfrac{1}{2}\ n\text{Mg}^{++}) \\ (\tfrac{1}{2}\ n\text{Ca}) \\ (\text{etc.}) \end{pmatrix} + \tfrac{1}{2}\ n\text{HgCl}_2 \rightleftharpoons$$

$$(\text{Bacterial cell}^{n-})\ (\tfrac{1}{2}\ n\text{Hg}^{++}) + \begin{pmatrix} (n\text{NaCl}) \\ (n\text{KCl}) \\ (\tfrac{1}{2}\ n\text{MgCl}_2) \\ (\tfrac{1}{2}\ n\text{CaCl}_2) \\ (\text{etc.}) \end{pmatrix}$$
should not grow

$(\text{Bacterial cell}^{n-})\ (\tfrac{1}{2}\ n\text{Hg}^{++}) + \tfrac{1}{2}\ n\text{H}_2\text{S} \rightleftharpoons$
$$(\text{Bacterial cell}^{n-})\ (n\text{H}^+) + \tfrac{1}{2}\ n\text{HgS}$$
should grow
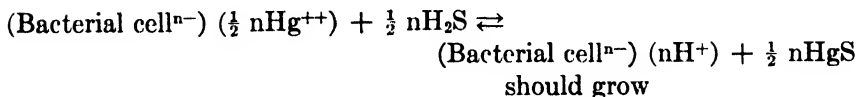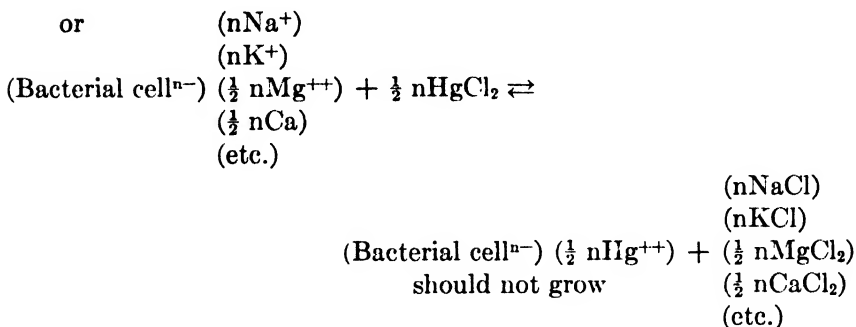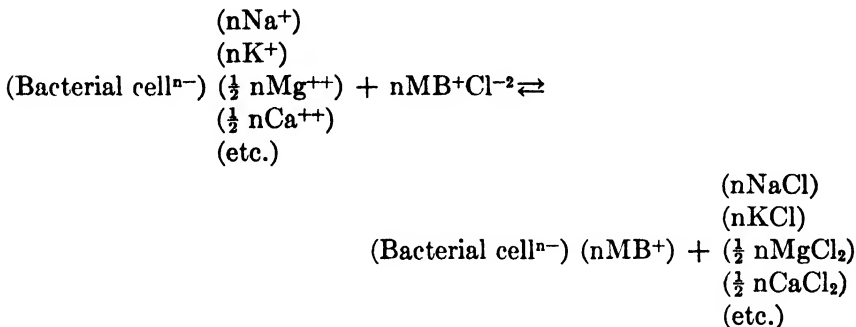
[1] MBCl refers to methylene blue chloride (Stearn and Stearn, 1930).

On the basis of the above theoretical consideration an effort was made to measure the cation adsorption capacity of *Escherichia coli* and other bacteria by four methods.

EXPERIMENTAL

The organisms were grown in large Blake bottles containing a suitable agar medium. After one to two days the bacteria were washed off the agar with 0.001 molar neutral $CaCl_2$ solution for H^+ adsorption and with neutral distilled water for methylene blue, mercuric chloride and magnesium adsorption.

H-adsorption. To simplify and reduce the cation to as nearly a single ionic system as possible, HCl was added to the aqueous suspension of the bacteria. The amount of HCl to be added

TABLE 1

Hydrogen adsorption capacity of E. coli and viability of the H-bacteria

TRIALS	ADSORPTION CAPACITY (M.E./100 GM. BACTERIA)	PER CENT VIABLE H-BACTERIA
1	54	100
2	35	88
3	57	100
4	38	89
5	53	100

was determined by titrating the neutral washed bacteria to a methyl red end point, or about pH 5.6–6.0. This amount of acid was arbitrarily designated as 1H and in suspensions containing about 0.2 to 0.3 per cent bacteria (dry weight) the HCl concentration approximated 0.0004 to 0.0008M, and HCl in slight excess of 1H was then added to the aqueous suspension of bacteria. The pH of such a suspension was approximately 5.0. The bacteria were then thrown out of suspension with the centrifuge and the unadsorbed HCl titrated. The H-bacteria were washed with distilled water and titrated with $Ca(OH)_2$. It was found that nearly all of the HCl added was adsorbed, and that the remainder was recoverable in the supernatant liquid.

The adsorption capacity as measured by this method ranged from 35 to 57 m.e. per 100 grams of bacteria as shown in table 1.

Plate counts carried out in quadruplicates showed no appreciable reduction in the number of viable bacteria by this treatment.

That this adsorption is purely a physico-chemical phenomenon, in no way depending upon the existence of a living protoplast, is indicated by the fact that bacteria killed by heating at 100°C. exhibited, within the limits of error, the same adsorption capacity. (See table 2). The adsorption capacity of *Azotobacter, Rhizobium,* and *Bacillus subtilis* compared favorably with that of *E. coli.*

Methylene blue adsorption. The slightly modified method of Kolthoff and Overholser (1939) was used to measure methylene blue adsorption by *E. coli.* Methylene blue, medicinal, of 88 per cent purity was used.[3] A standard solution of 0.01 M concentration was prepared and kept in a paraffined Pyrex flask.

TABLE 2

Adsorption capacity of living and dead bacteria

TRIALS	ADSORPTION CAPACITY (M.E./100 GM. BACTERIA)	
	Dead	Living
1	30	30
2	32	35
3	38	37
4	56	43

In adsorption studies this was added to washed bacterial suspensions so as to give concentrations of 0.0004 and 0.0016 M solution of methylene blue. This mixture was shaken thoroughly and then centrifuged. The amount of methylene blue not adsorbed by the bacteria was measured by comparing the supernatant liquid with a control solution. Quantitatively, the adsorption of methylene blue was, within the limits of error, the same for living and dead bacteria. The adsorption capacity of bacteria as measured with the 0.0004 M methylene blue varied from 6 to 14 m.e. per 100 gm. When the concentration of the methylene blue was quadrupled the absorption was increased as shown in table 3.

[3] Certified by Commission on Standardization of Biological Stains.

Influence of different cations on adsorption of methylene blue.
If the physico-chemical behavior of bacteria is similar to that of
other colloidal complexes, as regards adsorption and base ex-
change, then it should be possible to note a difference in the rela-
tive ease with which different cations adsorbed by the cell can
be replaced, depending upon the position of the cations in the
adsorption series.   To test this point, 1 ml. of 0.1 N NaCl, KCl,
CaCl₂, BaCl₂, and HgCl₂ was added, respectively, to 25 ml. of
suspensions of bacteria.   After allowing sufficient time for these
ions to occupy their positions in the adsorption sphere of the
bacteria, 1 ml. of a 0.001 M methylene blue was added to each
tube.   This   was   shaken   thoroughly   and   centrifuged.   The
amount of unadsorbed methylene blue was measured by com-

TABLE 3

Adsorption of methylene blue

TRIALS	ADSORPTION CAPACITY WITH FOLLOWING CONCENTRATIONS (M.E./100 GM. BACTERIA)	
	0.0004 M	0.0016 M
1	13	30
2	14	
3	6	24

paring with a control.   About 92 per cent of the methylene blue
was adsorbed by the Na and K systems, 75 per cent by Ca and
Ba systems, and 50 per cent by Hg systems.   Comparable re-
sults were obtained regardless of whether the experiments were
carried out on living or dead bacteria.

Replacement of adsorbed magnesium.   Magnesium sulfate was
added to a suspension of *E. coli* to equal approximately 0.001 M
solution.   After allowing sufficient time for the magnesium to
become adsorbed, the cells were washed until free of dissolved
magnesium.   To 25 ml. portions of the bacterial suspensions
were added, respectively, 1 ml. of 0.1 N NaCl, KCl, CaCl₂,
BaCl₂, MnSO₄, HCl.   These suspensions were shaken thoroughly
and then centrifuged.   The magnesium was measured by the
method of Baver and Bruner (1939).   The control showed a

very slight amount of replaced magnesium. Ions like Na and K, which are adsorbed slightly or not at all, replaced such a small amount of magnesium that it was not measurable by this method. Other ions which are more strongly adsorbed replaced the adsorbed magnesium. The values obtained by replacing the adsorbed magnesium compare favorably with those obtained by measuring the adsorbed hydrogen. These values are shown in table 4.

Adsorption of HgCl₂ by E. coli and its replacement. If the concept of adsorption of cations in bacterial metabolism is correct, as here advanced, the possibility of explaining the toxic effect of certain electrolytes upon a similar basis would not seem illogical. In fact, an arrangement of metallic ions in the order of their toxicity for bacteria (Salle, 1939) corresponds to the

TABLE 4

Replacement of adsorbed magnesium

TRIALS	AMOUNT OF ADSORBED MG. REPLACED BY VARIOUS CATIONS (M.E./100 GM. BACTERIA)						
	Control	Na	K	Ca	Ba	H	Mn
1	Very slight	Slight	Slight		16.5	26	
2	Very slight	Slight	Slight	38	38	50	38
3	Very slight	Slight	Slight	54	54		54

order of their adsorption ability by colloids (Kruyt, 1930, Jenny and Reitemeir, 1935).

Upon this basis it might be expected that a given bacterium could be subjected to such a concentration of a toxic cation as would prove lethal and that later, it should be possible before death has actually occurred, to displace the toxic ion with one less toxic and by this means preserve the life of the cell. However, if toxicity is correlated with adsorption ability, such displacement would be impossible without increasing the concentration of the exchange ion to such a degree as might render it also toxic. The same end can be attained by suspending the poisoned cell in a solution of an anion for which the toxic cation has a greater affinity than it does for the bacterial cell.

Stearn (1928) and Engelhardt (1922), "showed that staphylo-

cocci, which had been treated for 72 hours in a 1 per cent mercury bichloride solution would, after removal of the mercuric ion by precipitation as sulfide, grow."

A similar experiment was carried out on *E. coli*. To a washed suspension of the organisms containing about 0.2 per cent dry weight of cells, $HgCl_2$ solution was added to give approximately 0.0004 M concentration. After one hour, some of the suspension was diluted and then plated out. Less than 1 per cent of the cells grew. If the Hg ion had not been adsorbed, then in the dilution process the 0.0004 M solution would become a 0.000, 000,000,0004 M solution. Salle (1939) stated that Winslow and Hotchkiss (1922) have shown that 0.000,005 M $HgCl_2$ is not only not toxic but is definitely stimulatory to the growth of

TABLE 5

Adsorption of mercuric ion and its replacement

TRIALS	PER CENT VIABLE AFTER 60 MINUTES	
	Hg	Hg removed with H_2S
1	1.0	99
2	0	90
3	1.0	97

E. coli. When H_2S was bubbled through a portion of the same Hg-cell suspension, thereby removing the Hg-ion from the adsorption sphere of the organism, almost complete viability of the organism was observed. These data are given in table 5.

DISCUSSION

The data herewith presented are in agreement with the theoretical consideration upon which the experimental work was predicated. All common media contain various cations in solution. If a bacterial cell is placed in, or develops in such a medium, apparently there is an exchange of ions between the solution and cell until an equilibrium is established compatible with the energy of adsorption of the ions and their distribution in the solution. If an ion is used by the organism and becomes

an intimate part of the cell, it is possible that this process would remove an ion from the adsorption sphere. Consequently, a further shift in the equilibrium would be expected and another ion would be removed from the solution to replace the first.

In bacterial metabolism large amounts of CO_2 are liberated as waste products. The CO_2 in the presence of H_2O forms H^+ and HCO_3^-. The H^+ is adsorbed by the cell. A continuation of this reaction might result in a complete H-bacterial system. In the presence of other ions, however, an exchange of the H^+ for basic ions to equalize their distribution would be expected. Such an adsorption of cations by a bacterial cell from solution intimates that the cell may be able to concentrate and bind these ions as a store for future use.

The presence of an adsorption complex on the surface or in the interior of a bacterial cell indicates that some agent has set up an electrical force capable of holding these ions. A somewhat similar force undoubtedly orients the water molecules immediately surrounding the cell, thus creating a water hull in which the cell is encased. Each ion is likewise hydrated. Even though the ion may be adsorbed by the cell it is still in active motion, oscillating within its sphere of attraction.

The active places for attraction of the cations are not known. An approximate calculation of the number of H ions adsorbed by *E. coli* indicates that there are about 100,000,000 active sites per bacterial cell. Oxygen ions are sites of strong attraction for cations. It is well known that in proteins polar groups orient in the aqueous phase whenever possible. The presence of such groups in the cell or on the surface of the cell would produce an ionizable H which could be replaced by any cation as indicated in the following equation:

$$R - CH^-NH_2 - COOH = R - CH^-NH_2 - COO^- + H^+$$

Such active spots might account for the presence of a cation adsorption complex in a living cell. From the results it appears that when matter becomes living it does not lose its physico-chemical properties such as the power to adsorb various materials.

CONCLUSIONS

On the basis of the results obtained in this investigation, the following assumptions seemed well-founded.

1. *Escherichia coli* adsorbed cations and the magnitude of this capacity was measured. The ability of *E. coli* to adsorb cations was demonstrated by four methods.

2. Other bacteria adsorb cations.

3. A proposed mechanism of adsorption is advanced.

4. The adsorbed cations are exchangeable.

The writer is indebted to L. D. Bushnell and P. L. Gainey, Bacteriology Department, Kansas State College, for their suggestions.

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PHYSICO-CHEMICAL BEHAVIOR OF SOIL BACTERIA IN RELATION TO THE SOIL COLLOID[1]

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The natural environment of soil bacteria is highly complex from a nutritional standpoint. The colloidal clay fraction is the most active constituent of the soil in controlling a balance of the mineral nutrients, since it is this portion of the soil which adsorbs the various essential ions (Albrecht and McCalla, 1938). The colloidal clay is a hydrated aluminum silicate composed of superimposed layers of aluminum and silicon, bound together through oxygen bridges (Ford, Loomis, and Fidion, 1940). When the sheets of the clay crystal become hydrated, they expand and the clouds of adsorbed ions vibrate around and between these sheets. The nature and the interpenetration of the adsorbed ion have a marked influence on the character of the colloidal clay.

Since bacteria are in the upper range of colloidal limits, it seems perfectly logical to expect them to assume some of the physical characteristics of colloids. Furthermore, in a previous paper (McCalla, 1940) it has been shown that they do behave as colloids in so far as cation adsorption is concerned. McGeorge, 1932, also stated from his work on the adsorption of minerals by alfalfa that the adsorbed mono and di-valent ions were exchanged in stoichiometric proportions. Hence, in a study of the influence of the natural environment on the soil bacteria the relationship between these two colloidal substances—one a living organized entity and the other an inorganic colloid—should be considered.

It is the purpose of this paper to show that the mineral needs of

some common soil bacteria are probably satisfied by either solution or contact exchange of adsorbed ions between the bacterial cell and soil colloid, the degree of exchange of adsorbed ions being governed by the relative and differential adsorbability of the essential ions by these two colloidal systems. It is a well known fact that ions vary considerably in their adsorbability by colloids (Docking and Heymann, 1939). In a colloidal system containing two ions the play of forces determining which of the two ions will occupy the adsorption sites of the colloid is represented by the following equation, (Jenny, 1936).

$$W = \frac{\left[S + N_w + N_b\left(1 - \frac{V_w}{V_b}\right) \right] \pm \sqrt{\left[S + N_w + N_b\left(1 - \frac{V_w}{V_b}\right) \right]^2 - 4SN_w\left(1 - \frac{V_w}{V_b}\right)}}{2\left(1 - \frac{V_w}{V_b}\right)}$$

Where

W = number of electrolyte ion w particles adsorbed or electrolyte ion b released at equilibrium.

S = saturation capacity.

V_w, V_b = oscillating spaces of the adsorbed ions.

N_w = number of w particles added initially (electrolyte ion w).

N_b = number of b particles added initially (electrolyte ion b).

As other ions are added to the system the relationships become increasingly more complex. If, as in the case of a living bacterial cell, the adsorbed ion was removed by growth, it would be necessary for a new set of reactions to occur until equilibrium was again established. Knowledge of the laws governing the interrelationship of the ions in the bacterial cell and soil colloid should furnish a logical basis for evaluating bacterial utilization of mineral nutrients in the soil.

Without the means of some force for attracting the ions adsorbed by the soil colloid, it seems probable that a bacterial cell would be unable to grow. If, however, bacteria do behave as

colloids and exhibit base adsorption phenomena, it would be expected that when the soil bacteria grow in the soil, they can then compete with the soil colloid for the solution ions. If there were no excess of ions in solution, then the colloid having the greatest amount of adsorbed ions would be expected to yield these to the other until an equilibrium was established. Normally, the flow of ions would be to the bacterial cell; but, conceivably, in a soil deficient in bases the process might be reversed. In such a system not only would growth not be expected to occur but death would ensue. If the bacterial cell utilized these adsorbed ions in growth and they became an intimate part of the cell through the growth process, this would remove them from the adsorption sphere of the bacteria and there would be a further shift of ions from the soil colloid to the bacterial cell. Assuming the presence of sufficient amounts of all other nutrients and proper elimination of waste products, growth might be expected to proceed until there was an ultimate equilibrium in ions between the two systems.

<div align="center">EXPERIMENTAL</div>

The influence of a colloidal clay complex upon soil bacteria was studied experimentally as follows. The base adsorption capacity of Putnam colloidal clay[2] was measured by converting the clay colloid with different adsorbed bases into an H-clay by dialyzing under an electrical potential of 110 volts and then titrating the H-clay with a base. The adsorption capacity was 67 m.e. (milligram equivalents) per 100 grams of clay. The method of measuring the cation adsorption capacity of bacteria has been given in a previous paper (McCalla, 1940). The $Ca(OH)_2$ titration curves of H-legume bacteria and H-clay are presented in figure 1. Some of the common soil bacteria—*Bacterium globiforme, Corynebacterium simplex, Bacillus elegans, Bacillus alpinus,*[3] *Rhizobium meliloti, Azotobacter chroococcum*—were used

to determine whether soil bacteria would adsorb cations. Gibson, 1939, has emphasized the numerical importance of the *Corynebacterium* complex and *B. globiforme* in the soil. Conn *et al*, 1928, 1940, have frequently used *B. globiforme* as a test organism in soil microbiology studies.
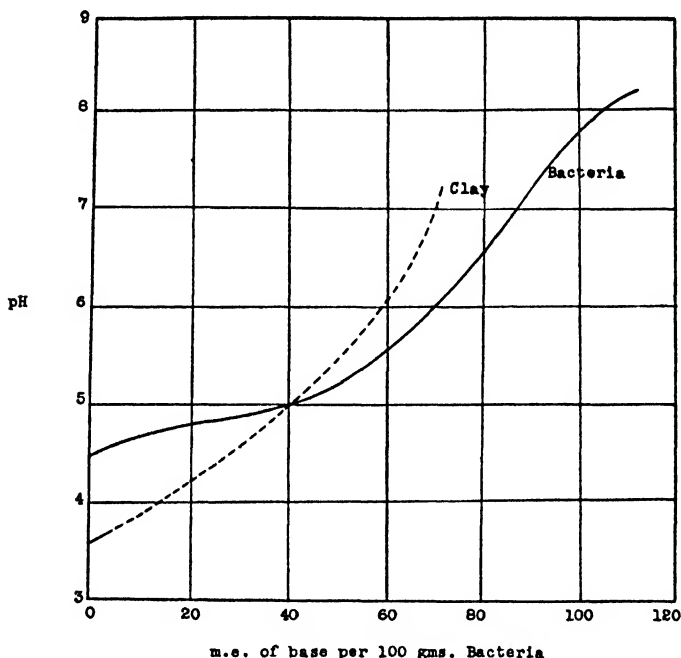
FIG. 1. CALCIUM HYDROXIDE CURVES FOR COLLOIDAL CLAY AND LEGUME BACTERIA

Hydrogen and methylene blue adsorption

The hydrogen adsorption capacity of the soil bacteria was determined by growing them on nutrient agar in large Blake bottles for 2 to 4 days at room temperature, suspending the cells in distilled water, thoroughly washing, and then converting them into H-bacteria by adding enough 0.01 N HCl to saturate the displacing capacity of the bacterial system. The adsorbed H^+ was measured by titrating the washed H-bacteria with $Ca(OH)_2$. In order to determine the viability of the H-bacteria,

plating in quadruplicate was made with untreated and H-bac-teria. Almost complete viability of the H-bacteria was evident.

Adsorption of methylene blue by the soil bacteria was deter-mined by adding a known concentration of methylene blue[4] solu-tion of 88 per cent purity, medicinal, to a washed bacterial sus-pension, throwing the cells out of suspension by means of an angle centrifuge and measuring the unadsorbed methylene blue by colorimetric method. The adsorption value was expressed as m.e. per 100 grams of bacteria dried at 105°C. The adsorp-

TABLE 1

The adsorption of hydrogen and methylene blue by soil bacteria

ORGANISM	TRIALS	M.E. ADSORPTION PER 100 GRAMS		PER CENT VIABLE OF H-BACTERIA
		Methylene blue	Hydrogen	
Bacterium globiforme	1	20.0	21.0	100
	2	20.0	20.0	94
Corynebacterium simplex	1		23.3	100
	2	20.0	16.8	80
Bacillus elegans	1	15.0	26.0	99
	2	16.0	25.0	85
Bacillus alpinus	1	23.6	49.0	92
Azotobacter chroococcum	1	30.0	21.0	
	2	35.0	25.0	

tion value of H^+ was similar to that obtained with methylene blue as indicated in table 1. In all instances there was some adsorption of H^+ or methylene blue$^+$.

The replacement of adsorbed methylene blue with different metallic ions

If bacteria adsorb cations, then a relative adsorbability of different ions varying as their adsorption energy should be evident. In order to get a comparative view of the energy of

[4] Certified by commission on standardization of biological stains.

adsorption of the different essential ions, determinations were made to see how much of some single adsorbed ion would be removed when equivalent ion concentrations of the various minerals were added. Methylene blue was used as the adsorbed ion because its quantitative removal may be measured colorimetrically. Gieseking and Jenny, 1936, showed that methylene blue could be adsorbed by a soil colloid and then replaced with some ion. Methylene blue was added at the rate of 20 m.e. per 100 grams to washed cells of *B. globiforme* grown on calcium gluconate agar (Albrecht and McCalla, 1937a). The cells were

TABLE 2

Replacement of adsorbed methylene blue with different ions

ION	IONIC RADIUS* OF CATION A* UNIT	PER CENT METHYLENE BLUE REPLACED		
		0.5S	1S	5S
Na	0.98	2.90	3.12	3.68
NH₄	1.46	3.34	3.68	4.30
K	1.33	4.16	4.76	5.26
Mg	0.78	6.76	6.92	
Ca	1.02	5.00	7.05	11.1
Ba	1.43	5.76	8.34	12.5
Mn		7.15	8.70	12.5
Al	0.57	25.00	33.40	
Fe	0.67	31.40	46.2	54.6
H	0.28	21.0	66.4	

* Data from Baver and Hall, 1937. Emeléus and Anderson, 1939.

washed to remove the unadsorbed methylene blue. To determine the amount of adsorbed methylene blue a given ion would replace, the exchanging ion was added in symmetry concentration (1S)[5] and the amount of methylene blue released from the bacterial cell was measured by comparing with a standard in a colorimeter. The symmetry concentrations of the different ions used were 0.5S, 1S and 5S. The results are listed as symmetry values. For example, if one-half of the adsorbed methylene blue is displaced by an ion, the symmetry value is recorded as

[5] Symmetry concentration is obtained when the number of replacing ions added is equal to the number of adsorbed ions.

50 per cent. The displacing ions used were Na^+, K^+, NH_4^+, Ca^{++}, Ba^{++}, Mg^{++}, Mn^{++}, Fe^{+++}, Al^{+++}, and H^+. All were prepared as 0.01 N solutions as chlorides except Al, Mn and Mg, which were prepared in the form of sulfates, and added to a suspension of *B. globiforme*. A typical set of data given in table 2 place the adsorption series as follows in replacing power: $Na < NH_4 < K < Mg < Ca < Ba < Mn < Al < Fe < H$. This experiment was repeated using different organisms. The replacement value, however, varied with the type and age of organism and kind of media upon which the culture was grown. The adsorp-

TABLE 3

The relative growth of soil bacteria in solution and colloidal media

ORGANISM	TRIALS (MEAN OF TRIPLICATES)	BACTERIA IN MILLIONS PER ML.	
		Calcium gluconate	Colloidal clay
Rhizobium meliloti..	1	490	3,980
	2	284	2,250
	Mean	387	3,115
Bacterium globiforme	1	279	937
	2	5	549
	Mean	142	743
Corynebacterium simplex... ..	1	787	1,433
Bacillus alpinus	1	4	42
	2	14	103
	Mean	9	72

tion series, nevertheless, retained the same general sequence as might be expected from the charge and size of the ion.

Biochemical relation of soil bacteria to soil colloid

The effect of the colloidal clay upon the growth of the soil bacteria was measured quantitatively by growing the organisms in an ordinary calcium gluconate medium and in this same medium plus H-clay colloid in 2.5 per cent concentration. The acidity of the calcium gluconate medium, caused by the addition of the H-clay, was neutralized by the addition of equal quantities

of KOH and Ca(OH)$_2$. The composition of the calcium gluconate medium was as follows: Na as NaCl 3.34 m.e.; Mg as MgSO$_4$ 3.33 m.e.; K 2.66 m.e., and P 4.00 m.e. as K$_2$HPO$_4$ calcium gluconate 1.5 gram; sucrose 10 grams; NH$_4$NO$_3$ 0.1 gram; and distilled water to make 1000 ml. Each trial carried out consisted of triplicate tubes containing 25 ml. of the medium inoculated with a measured suspension of the actively growing soil organism used. In order to make the conditions in such media as favorable as possible, a current of sterile air was forced through the cultures for three to four days after which quantitative plate counts were made. An example of the data collected in this manner is presented in table 3. From these data it is evident that the clay-containing medium was very much more favorable for growth than the ordinary mineral liquid medium. Attention has already been called to the influence of a colloidal clay complex upon various biochemical phenomena (Albrecht and McCalla, 1937b; McCalla, 1937; McCalla, 1939; Conn and Conn, 1940).

DISCUSSION

The fact that the various soil bacteria made better growth in the presence of colloidal clay indicates that there is a marked difference in the effect of the two media. Conn and Conn, 1940, also have demonstrated the value of colloidal clays as growth promoters. The addition of the clay, apparently inert as a nutritive material, has in some way stimulated the growth of the soil bacteria. Broughton, 1940, verified previous findings that clays were of value as commercial catalysts. Since the addition of the colloidal clay to the mineral medium increased bacterial growth, it would not seem impossible that the clay functioned catalytically in speeding up biochemical reactions, either by providing for a more efficient utilization of nutritive material or by decreasing toxic effects of waste products by adsorbing them.

In the soil the colloidal material intermingled with coarser minerals forms definite structures. Some soil scientists suggest that soil structure is a result of an edge-to-edge contact of the colloidal platelets. Under these conditions, if a bacterial cell

were located at one side of the soil colloidal structure, the ions adsorbed at different positions on the colloidal particles could migrate on the colloidal surfaces (Jenny and Overstreet, 1939) to the cell as they were utilized.   In ordinary laboratory media used to cultivate bacteria, water is the major constituent and the nutritive material is about 1 to 5 per cent; but under normal conditions in the soil the solid material constitutes about 80 per cent whereas the water is present in about 20 per cent concentration around and between the particles of the soil.   Under
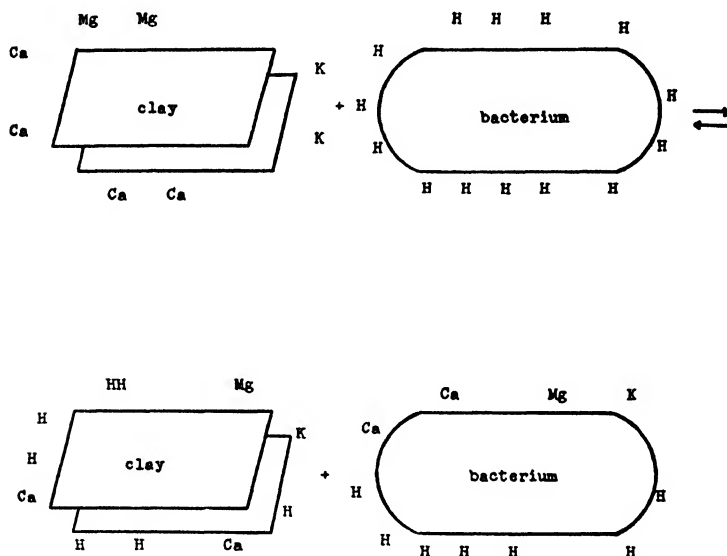
Fig. 2.   Mechanism of Ion Exchange Between Soil Bacteria and Colloidal Clay

these circumstances the bacteria probably live in the water films which adhere to the surface of the colloid containing the adsorbed ions.   The bacterial cell is undoubtedly in close proximity to the soil particles and assuming that the bacteria may adsorb ions and hold some of them in the outer surface of the cell, this would permit contact exchange of adsorbed ions.   Ions with large oscillating volumes would overlap, and exchange between systems could readily take place.   Other ions which are strongly adsorbed would not be expected to wander far from the surface

of the colloid. On the basis of the displacement of adsorbed methylene blue the ions would be expected to be adsorbed by the bacteria from the soil colloids in the following series: $H > Al > Fe > Mn > Ba > Ca > Mg > K > NH_4 > Na$.

From the foregoing facts and theoretical considerations it is suggested that in the adsorption of nutrients from the soil by the bacteria, and possibly by living cells in general, an exchange of adsorbed bases takes place between the bacteria and soil colloid as depicted in figure 2. In bacterial metabolism large amounts of carbon dioxide and water are formed. In the presence of H_2O H-ions are produced from the carbon dioxide which may be adsorbed at the cell's surface. When a colloidal clay particle, saturated with adsorbed bases contacts a bacterium saturated with H-ions, an exchange of ions takes place until an equilibrium is reached. As this equilibrium is upset by the more complete utilization of the adsorbed basic ions in bacterial metabolism a further exchange may take place, the colloidal clay functioning as a constant reservoir for basic ions utilized in the growth of such organisms.

CONCLUSIONS

1. Soil bacteria adsorb cations.

2. Adsorbed methylene blue may be replaced by other cations. The degree of adsorption of the replacing ion determines how much methylene blue is replaced.

3. The order of the adsorption series is as follows: $Na < NH_4 < K < Mg < Ca < Ba < Mn < Al < Fe < H$.

4. Colloidal clay stimulates the growth of soil bacteria.

5. It is proposed that soil bacteria obtain their mineral needs by contact exchange of adsorbed ions between the bacterium and clay particles.

6. A series of equilibrium reactions occur between soil colloid and bacteria depending upon relative adsorbability of the ions by these two systems.

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A PRELIMINARY INVESTIGATION INTO THE ANTIGENIC CHARACTERS OF SPORE-FORMING, AEROBIC BACTERIA

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PART I

The systematology of the spore-forming aerobic bacteria has been examined by a number of investigators, in particular by Gottheil (1901); Neide (1904); Chester (1907); Ford and his collaborators (1916); Conn (1930). So far, however, only the biological characters of the bacteria have received attention, while no comparative serological investigations have been carried out. Isolated serological experiments, for instance the one made by Nyberg with *Bacillus mycoides* and Shorb and Bailey's, and With's (1935) demonstration of Forssman's antigen, do not render possible a systematic serological classification. The presence of the F-antigen has so far only been studied in regard to *Bacillus cereus* and *Bacillus subtilis*. It is not established, however, whether this character is common to all *B. cereus* stems as distinct from *B. subtilis*. An interesting difference between bacteria of the type mentioned above is seen in Aminoff's work (1939) on their power to influence originally WaR-negative sera to the effect that the latter, after infection, are capable of arresting the hemolysis. The above mentioned investigation yielded no definite group classification although bacteria belonging to the same biological groups in most cases behaved in the same manner.

Mention may be made of the work of Krauskopf and McCoy (1937) on the antigenic content of the spores of *Bacillus niger*. Although their studies were not directed toward establishing the

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[1] Part I describes the experiments made by Olof Sievers, part II those made by Bo Zetterberg.

systematic position of the organism in question, the results showed clearly that with spore-producing organisms there is a close serological relationship between the spore and the vegetative cell. A striking finding was the demonstration, by absorption methods, that spores may contain the flagellar H antigen.

With the intention of investigating the question of the possible antigenic structure of these bacteria and the possibility of separating the different groups from one another by the aid of the latter I made a number of immunizations. The strains investigated were kindly placed at my disposal by Mrs. M. Aminoff, Fil. Kand. The experiments included *Bacillus mesentericus, Bacillus cereus, Bacillus mycoides, Bacillus vulgatus* and *Bacillus subtilis.* The characteristic biological characters of bacteria belonging to the above-mentioned species have been described by Aminoff (1939) in her work on the power of these bacteria to affect sera in the Wassermann test.

Method of immunization

In preparing the antigen for the injections these bacteria were cultivated on ordinary agar and suspended in physiological salt solution. Non-emulsive portions are easily obtained in this process, which cannot be used in the injections. In order to obtain as homogeneous an emulsion as possible cultures of about 12 hours were used. The bacteria were transferred repeatedly, approximately every 12th hour, to new agar and re-suspended only after 3 to 4 reinoculations. The emulsion obtained in this manner remained remarkably homogeneous after filtration through cotton-wool and contained an extremely small number of spores. A suspension of older cultures, on the other hand, always holds an abundance of spores. The first experiments indicated that the test rabbits yielded antibodies more easily after injection of young homogeneous emulsions. In this connection I shall not enter on a direct comparison between results obtained by emulsions of cultures of unequal age. I only wanted to stress at this point the kind of material with which the experiments were made.

The rabbits received intravenous injections in increasing quantities at intervals of 3 to 4 days, and blood serum was se-

cured after experiments had shown complement-fixing antibodies against the homologous strain. The strains with which the immunizations were carried out were arbitrarily selected from among the bacterial strains placed at my disposal.

Complement-fixation

On account of a tendency on the part of the bacteria to agglutinate spontaneously, I was forced to test the reaction power of the antisera by means of fixation of the complement. For the same reason I had to abandon the agglutination process in subsequent experiments. The first reading after 1 to 2 hours often showed that antisera had an agglutinative effect on the homologous bacteria. After 6 to 20 hours, heterologous strains were also strongly agglutinated and, further, the strains which reacted within 1 to 2 hours were usually agglutinated in the salt solution control. The complement fixation tests, on the other hand, yielded a clearer reaction.

The following record describes one of the experiments made:

0.1 ml. of *B. vulgatus* 32 antiserum in decreasing quantities (volume 0.1 ml.) and 0.1 ml. suspensions of the following bacteria

a		No. 14b
b	*B. subtilis*	No. 15
c		No. 19b
d		No. 20b
e		No. 19a
f	*B. mycoides*	No. 21a
g		No. 22
h		No. 24a
i		No. 32
k	*B. vulgatus*	Nc. 42
l		No. 38
m		No. 57

were incubated for one hour with 0.1 ml. of guinea pig complement. After addition of 0.1 ml. of amboceptor dilution, $2\frac{1}{2}$ times the dose causing total hemolysis, and 0.1 ml. of 5 per cent suspension of sheep blood corpuscles, the hemolysis given in table 1 was observed.

The record shows that *B. vulgatus* 32 antiserum only reacted with two of the *B. vulgatus* strains tested. Neither *B. cereus* nor *B. mycoides* yielded any reaction. A repeated experiment with decreasing quantities of bacteria and constant quantity of antiserum (1:10) yielded the same result.

I also tested one *B. mycoides* 24 antiserum and one *B. cereus* 14b antiserum. The first sample reacted with the four *B. mycoides* strains tested (19a, 21a, 22 and 24a) and with *B. vulgatus* 32 and 38 respectively, but not with *B. vulgatus* 42 and 57 respectively, and not with *B. cereus* 14b, 15, 19b, and 20b. Here

TABLE 1

B. VULGATUS ANTISERUM IN DECREASING QUANTITIES	HEMOLYSIS OF BLOOD CORPUSCLES OF SHEEP BY MEANS OF AMBOCEPTOR AND COMPLEMENT AFTER PREPARATORY TREATMENT OF THE LATTER WITH B. VULGATUS 32 ANTISERUM AND											
	B. subtilis				*B. mycoides*				*B. vulgatus*			
	14b	15	19b	20b	19a	21a	22	24a	32	42	38	57
1. 1:10 0.1	−	−	−	−	−	−	−	−	+	−	(±)	−
2. 1:20 0.1	−	−	−	−	−	−	−	−	+	−	(±)	−
3. 1:40 0.1	−	−	−	−	−	−	−	−	+	−	+	−
4. 1:80 0.1	−	−	−	−	−	−	−	−	+:	−	+	−
5. 1:160 0.1	−	−	−	−	−	−	−	−	+:	−	+	−
6. 1:320 0.1	−	−	−	−	−	−	−	−	−	−	(±)	−
7. 1:640 0.1	−	−	−	−	−	−	−	−	−	−	(±)	−
8. 1:1280 0.1	−	−	−	−	−	−	−	−	−	−	−	−

The hemolysis was denoted by: + = no hemolysis; +: = small traces of hemolysis; (+) = traces of hemolysis; ± = slight hemolysis; (±) = moderate hemolysis; −? = strong hemolysis; −(?) = almost complete hemolysis; − = complete hemolysis.

the antiserum consequently reacted in some measure also with biologically heterologous strains. The record given above showed that, conversely, no reaction occurred between *B. vulgatus* 32-antiserum and *B. mycoides*. These two species of bacteria, *B. mycoides* and *B. vulgatus*, to all appearance cannot therefore be looked upon as serologically identical. The results of the biological and serological investigations tally with one another in regard to these two types of bacteria.

As for *B. cereus* 14b antiserum, it reacted with *B. cereus* 14b, 15, and 19b respectively, but not with *B. cereus* 20b. Further,

B. vulgatus 32, 38 and 57 respectively, and *B. mycoides* 22 reacted with this antiserum, whereas the other bacteria tested (see above) did not react.   This antiserum, from the point of view of specificity, did not yield as favorable results.   It is worthy of note, however, that even though *B. cereus* 14b antiserum reacted with a number of bacteria of other types, the *B. cereus* strains did not react with the other two antisera.

Precipitation-tests

The account given above seems to indicate that *B. vulgatus* and *B. mycoides* differ serologically from one another and that both differ from *B. cereus*.   In this connection I shall describe a few other precipitation experiments.   *B. cereus* 14b, *B. vulgatus* 32, *B. mesentericus* 41, *B. mycoides* 24a and *B. subtilis* 28 were cultivated in the manner described above on ordinary agar. Cultures of about 12 hours' standing were suspended in 0.5 per cent carbolic salt solution and filtered through sterile cotton. Only isolated spores could be observed in these emulsions; in some samples they were absent.   The emulsions were made extremely thick and were then kept for four months at a temperature of 4°C.   During this time, most of the bacteria went into lysis.   The flask with *B. subtilis*, for instance, contained no bacteria, whilst traces of bacteria were observable in the flasks holding *B. mesentericus*, *B. mycoides* and *B. vulgatus*.   In the flask with *B. cereus* a large number of bacteria were still present, although noticeably less than four months previously.   All these emulsions were centrifuged until the centrifugates seemed to be wholly clear.   The latter were then tested with different antisera by the process of precipitation.

The table below shows some of the precipitation tests.   To 1 ml. of the different autolysates in decreasing quantities, dilution in physiological salt solution, 0.1 ml. of the different undiluted antisera was carefully added.   The results observed after 25 minutes are recorded in table 2.

An examination of the table shows a marked specificity in the different antisera.   Antisera 8 and 34, for instance, only reacted with autolysates of the corresponding homologous bacterium.

TABLE 2

Autolysate

TUBE	DILUTION	B. MYCOIDES 24a ANTISERUM NUMBER 10					B. MESENTERICUS 41 ANTISERUM NUMBER 8					B. VULGATUS 32 ANTISERUM NUMBER 34					B. SUBTILIS 28 ANTISERUM NUMBER 7					B. CEREUS 14b ANTISERUM NUMBER 1411				
		B. subtilis 28	B. mycoides 24a	B. mesentericus 41	B. vulgatus 32	B. cereus 14b	B. subtilis 28	B. mycoides 24a	B. mesentericus 41	B. vulgatus 32	B. cereus 14b	B. subtilis 28	B. mycoides 24a	B. mesentericus 41	B. vulgatus 32	B. cereus 14b	B. subtilis 28	B. mycoides 24a	B. mesentericus 41	B. vulgatus 32	B. cereus 14b	B. subtilis 28	B. mycoides 24a	B. mesentericus 41	B. vulgatus 32	B. cereus 14b
1	1:2	±	+	—	+	—	—	—	+	—	—	—	—	—	+	—	+	—	—	+	—	+	—	—	+	+
2	1:4	—	+	—	+	—	—	—	+	—	—	—	—	—	+	—	+	—	—	+	—	+	—	—	+	+
3	1:8	—	+	—	+	—	—	—	+	—	—	—	—	—	+	—	+	—	—	+	—	—	—	—	+	+
4	1:16	—	+	—	+	—	—	—	+	—	—	—	—	—	+	—	+	—	—	—	—	—	—	—	—	+
5	1:32	—	+	—	—	—	—	—	+	—	—	—	—	—	+	—	+	—	—	—	—	—	—	—	—	+
6	1:64	—	+	—	—	—	—	—	+	—	—	—	—	—	+	—	—	—	—	—	—	—	—	—	—	—
7	1:128	—	+	—	—	—	—	—	+	—	—	—	—	—	+	—	—	—	—	—	—	—	—	—	—	—
8	1:256	—	—	—	—	—	—	—	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
9	1:512	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

The other three also reacted with *B. vulgatus* and antiserum 1411, also somewhat with *B. subtilis* autolysate. Quantitatively, however, there was in these cases a marked difference between the autolysates of the homologous and of the heterologous bacteria. The different autolysates need not be equally strong, it is true, as it is impossible to decide to what extent the antigen principle has passed into the solution. The strong reactions with homologous antisera, however, indicate that in each separate case a sufficient amount of antigen is present in the solutions tested for a comparison to be justified.

With the exception of antiserum 1411 (*B. cereus* 14b), the antisera used in the precipitation were not the same as had earlier been used in the complement fixation. The two different *B. vulgatus* antisera yielded the same result in the two experiments. The same applies to the two *B. mycoides* antisera: both reacted strongly with the homologous bacterium and to some extent also with *B. vulgatus*.

The experiments described were rather few in number, but the results seem to indicate the probability that a difference is present between the antigenic structures of the different groups of bacteria. Only a few arbitrarily chosen strains have now been tested and it remains to be seen whether a larger number of bacteria within the different groups behave in the same manner.

PART II

In the preliminary experiments made by Sievers on the antigens of spore-forming, aerobic bacteria only a small number of strains were tested. The results indicate, that, to all appearance, a difference is present, even though, as stated by Sievers, it is too early to decide whether a serological accord is present within each of the different species. In order to try in some measure to illustrate this I tested a number of strains (for details, see the tables) with some antisera. (An account of the technique adopted in the immunizations is given in part I.)

The technique adopted in the complement fixation tests was identical with the one used by Sievers. All the experiments were made quantitatively and the auto-inhibitory power of the

bacterium suspension was determined in all cases. Tests were made both with a fixation time of one hour at 37°C., and of one hour at 4°C. The results were practically identical. A comparison of fixation at room temperature and at 37°C. showed that the latter temperature was more suitable in these experiments. A combination of room temperature followed by 37°C. seemed to give a less favorable result than at 37°C. only.

Complement-fixation

From considerations of space the original records will not be given but only a few tabular surveys. The sign — denotes, that

TABLE 3

B. mycoides 24a antiserum, no. 11, and the indicated different species of bacteria

B. SUBTILIS NUMBER	REACTION	B. MYCOIDES NUMBER	REACTION	B. MESENTERICUS NUMBER	REACTION	B. VULGATUS NUMBER	REACTION	B. CEREUS NUMBER	REACTION
28	—	12a	+	9	—	18	—	8	—
48	—	13a	+	33	—	26	—	12b	—
54	—	14a	+	40	—	27	—	13b	—
68	—	19a	++	41	—	29	—	14b	—
		19c	+	43	—	32	—	15	—
		20a	+	44	—	38	—	16a	—
		21a	+			39	—	19b	—
		22	++			42	—	20b	—
		24a	++					21b	—
		25	+						

no inhibition of the hemolysis was observed, whereas ++, + and ± denote that such an inhibition was present. As indicated by the signs I have tried to grade the strength of the reaction. The lines of demarcation between the unequally pronounced reactions are naturally indistinct, but by this gradation the approximate strength of the reaction is seen in the tables.

It seems as though all the *B. mycoides* strains tested have an antigen in common which was lacking in the representatives of the other species examined. Another *B. mycoides* 24a antiserum no. 10, gave exactly the same reaction as the bacterial suspensions mentioned above. These two *B. mycoides* antisera seem to be

still more specific than the one tested by Sievers by the same technique. The joint results seem to indicate that *B. mycoides*, not only from a biological, but possibly also from a serological point of view, should be looked upon as a specific type in distinction to the other bacteria mentioned.

Table 4 shows a similar compilation of the results of tests with *B. mesentericus* antiserum.

As in the case of *B. mycoides* no reaction was here obtained with bacteria belonging to other species. As regards the *B. mesen-*

TABLE 4

B. mesentericus 41 antiserum, no. 9, and the indicated species of bacteria

B. SUBTILIS NUMBER	REAC-TION	B. MYCOIDES NUMBER	REAC-TION	B. MESEN-TERICUS NUMBER	REAC-TION	B. VULGA-TUS NUMBER	REAC-TION	B. CEREUS NUMBER	REAC-TION
28	−	12a	−	9	++	18	−	6b	−
48	−	13a	−	33	−	26	−	7b	−
54	−	14a	−	40	++	27	−	8	−
68	−	19a	−	41	++	29	−	12b	−
		19c	−	43	−	32	−	13b	−
		20a	−	44	−	38	−	14b	−
		21a	−	50	−	39	−	15	−
		22	−	51	−	42	−	16a	−
		24a	−	52	+	53	−	19b	−
		25	−	58	++	55	−	20b	−
				65	−	56	−	21b	−
				66	−	57	−		
						59	−		
						64	−		
						67	−		

tericus strains, no antigen factor common to them all was demonstrable by the fixation of complements. As yet we are only able to establish that all the reacting strains were of the *B. mesentericus* type. Another *B. mesentericus* 41 antiserum, no. 8, gave an identical result, except for the fact that *B. mesentericus* 52 did not arrest the hemolysis. In the case of strains not belonging to the *B. mesentericus* type, my results tallied with the reactions obtained in Sievers's precipitation tests.

Table 5 shows corresponding tests with a *B. vulgatus* 32 antiserum.

Almost half the number of *B. vulgatus* strains reacted with this antiserum. By the technique adopted it was not possible to demonstrate an antigen common to all these strains. Possibly one might here, as perhaps also in the case of *B. mesentericus*, by repeated experiments establish serologically characteristic subgroups. In distinction to the antisera mentioned above, this *B. vulgatus* 32 antiserum reacted with some strains of other types, one *B. subtilis*, two *B. mesentericus*, and four *B. mycoides* strains. As, with a reversed test arrangement, antisera against

TABLE 5

B. vulgatus 32 antiserum, no. 34, and the indicated species of bacteria

B. SUBTILIS NUMBER	REACTION	B. MYCOIDES NUMBER	REACTION	B. MESENTERICUS NUMBER	REACTION	B. VULGATUS NUMBER	REACTION	B. CEREUS NUMBER	REACTION
28	−	12a	++	9	−	18	+	8	−
48	−	13a	−	33	−	26	++	12b	−
54	+	14a	−	40	±	27	+	13b	−
68	−	19a	−	41	−	29	++	14b	−
		19c	++	43	−	32	++	15	−
		20a	−	44	±	38	−	16a	−
		21a	+			39	−	19b	−
		22	−			42	+	20b	−
		24a	−			53	−	21b	−
		25	+			55	−		
						56	−		
						57	−		
						59	−		
						64	−		
						67	+		

the three last mentioned types of bacteria and the different *B. vulgatus* strains, no reaction was obtained, we can hardly look upon all of the above mentioned reacting strains as serologically identical. The *B. vulgatus* antiserum tested by Sievers seemed to be specific to the extent to which it was tested.

The last table, 6, shows the results of tests with *B. subtilis* antiserum.

With the exception of the *B. vulgatus* strains, almost all the strains examined reacted. The earlier tables show, as opposed to the one given above, that only one of the four *B. subtilis*

strains reacted with another antiserum (*B. vulgatus* antiserum). This *B. subtilis* antiserum, now examined by me, was also tested by Sievers as shown in part I.   The results of my complement fixation tests and of the precipitation tests differ from one another. The outcome of the last mentioned reaction clearly indicates an antigen specificity as seen above in the case of the other species.

I had no *B. cereus* antiserum at my disposal and these bacteria, therefore, were not tested with homologous antiserum.   Sievers' experiments with precipitation together with my experience that the *B. cereus* strains do not react with any of the other types of antisera (with the exception of the above-mentioned *B. subtilis* antiserum) indicate that the *B. cereus* species might also possibly

TABLE 6

B. subtilis 28 antiserum, no. 7, and the indicated species of bacteria

B. SUBTILIS NUMBER	REACTION	B. MYCOIDES NUMBER	REACTION	B. MESENTERICUS NUMBER	REACTION	B. VULGATUS NUMBER	REACTION	B. CEREUS NUMBER	REACTION
28	++	20a	+	9	±	18	−	8	±
48	++	21a	±	33	±	26	−	12b	±
54	++	22	±	40	+	27	−	13b	+
68	++	24a	±			29	−	14b	−
		25	±			32	−	15	+
						38	−		
						39	−		
						42	−		

be serologically separated from the other types under discussion. Whether all *B. cereus* strains are to be looked upon as a homogeneous serological type, in the same sense as *B. mycoides*, cannot be decided on the basis of the present material.

An examination of Sievers' and my own results shows a fairly clear accordance.   My experiments have illustrated the possible serological unity of the species; moreover the serological difference observed by Sievers between the biologically different species has been further confirmed.

Summary

After injecting rabbits with *Bacillus subtilis*, *Bacillus mycoides*, *Bacillus mesentericus*, *Bacillus vulgatus*, and *Bacillus cereus*

respectively, antisera were obtained which gave complement fixation and precipitation (of autolysate) with the bacterial type which was used in the immunization in question.   Both in the immunizations and in the complement fixation tests, cultures of about 12 hours' standing, suspended in physiological salt solution, were used.

Agglutination tests were rendered impossible by the marked tendency to spontaneous agglutination on the part of the bacteria.

The different species separated by their different biological characters (Aminoff, 1939) in the preliminary examinations now made seem to be serologically separated, at least to some extent. The precipitation experiments with autolysates of a small number of strains speak in favor of the presence of a specific antigen structure in the different types.   The results of the complement fixation tests with a fair number of strains point in the same direction even though the specificity does not appear equally clear.

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A DESCRIPTION OF SOME COLOR VARIANTS PRO-DUCED BY SERRATIA MARCESCENS, STRAIN 274

MARY I. BUNTING

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Many workers have recognized the theoretical advantages to be derived from the use of a fast-growing, pigmented organism such as *Serratia marcescens* in the study of bacterial variation. Wasserzug (1888), Scheurlen (1896), Hefferan (1903), Rettger and Sherrick (1911), Eisenberg (1914), Breed and Breed (1927), Daddi (1932), Reed (1937) and others have reported observations on the appearance of more or less stable color variants of this species. These observations, however, have thrown little light on the mechanisms involved in the production of such variants. Some series or coincidence of events must be responsible for the cellular changes that are so conspicuous, but as yet so little is known concerning the rates at which variants arise, or the effects of environmental factors on rates of variation, that the nature of these events remains obscure.

It has been the writer's object to approach the problem of color-variation in *Serratia* in a quantitative manner, and to attempt to measure the frequency with which variants are produced under different environmental conditions. The work reported in this paper[1] is of a preliminary nature and is concerned with the selection of a strain suitable for intensive study, the development of a special medium, and the recognition and characterization of specific variant types.

Pigmented strains of *Serratia* were found to differ greatly both in color and in the frequency with which they produced paler variants. These differences proved so confusing that it was

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[1] These studies were begun at Bennington College in 1937 and continued at Goucher College through 1937–38.

finally decided to confine the first series of experiments to one highly pigmented and rather unstable strain, ⚹274 of the American Type Culture Collection. The morphology of the cells of this strain was not typical; in addition to short rods there were many as long as 6μ, and filaments ranging from 10μ to 30μ. The cells were gram-negative, motile and non-capsulated. Growth in peptone broth was turbid; a bright orange-red pigment was produced in the surface layers. Gelatin was liquefied promptly. Milk was made slightly acid, curdled and later digested. Acid, but not gas, was noted in fermentation tubes with glucose, sucrose and mannitol; no acid was produced from lactose. The majority of colonies on beef-extract peptone agar were circular, raised, smooth and bright orange-red; paler, more translucent variants were common; rough and mucoid variants rather rare. As reported by Reed in 1937, there was no correlation between pigmentation and colony type.

In order to study the production of color variants in a quantitative manner, it was essential to be able to distinguish specific variants. On the ordinary peptone beef-extract agar, this was not possible as the colony colors varied almost continuously from orange-red to white. Considerable effort was spent in developing a simple, easily reproducible synthetic medium which would permit good growth of *Serratia marcescens* in broth, and would give well differentiated colonies on agar plates. The medium which was finally chosen contained, per liter of distilled water: glycerol 5.0 grams, ammonium citrate 5.0 grams, $K_2HPO_4 \cdot 3H_2O$ 10.0 grams, $MgSO_4$ 0.5 gram, NaCl 0.5 gram, ferric ammonium citrate 0.05 gram. After autoclaving for ten minutes at 120°C. a clear broth buffered at pH 6.9 was obtained. When a solid medium was desired 1.5 per cent agar was added before autoclaving. Glycerol was used rather than glucose, because it was more heat-stable in the presence of the large amounts of phosphate which were employed to maintain the pH near neutrality during the growth of the cultures, and because its use resulted in the production of very highly pigmented red colonies. The ammonium ion proved to be a satisfactory source of nitrogen. Citrate was added to keep the inorganic salts in solution, to

stimulate growth (a property which it and other organic acids such as propionic and lactic were found to possess), and to aid in the maintenance of a neutral reaction. It may be noted that, although the addition of citrate decreased the lag phase in synthetic media containing glycerol or glucose, the initiation of growth was very slow indeed when citrate was the only available source of energy. Magnesium and iron were found to be essential to the growth and pigmentation of this strain of *Serratia*.

Reasonably rapid growth of strain 274 was observed in the synthetic ammonium citrate medium. In pour plates colonies became visible in 24 hours at 30°C. In 48 hours the surface colonies were from 1 to 3 mm. in diameter, and were brilliantly pigmented. The deep colonies were smaller and were colorless except in very crowded plates.

Superficial examination of colonies on three-day-old plates indicated that although the colony colors varied from dark red through different shades of pink to white, the variations were abrupt rather than continuous. The majority of the colonies were dark red (oxblood red[2]), a few were light red (bordeaux[2]), a large percentage were bright pink (eugenia red[2]), many were pale pink (hydrangea pink[2]), and a few were white.

It was interesting, in view of the fact that Reed had reported better color differentiation on acid than on alkaline media, to note that the beef-extract peptone agar was rendered distinctly alkaline by the growth of the organisms, whereas the pH of the synthetic medium remained near neutrality. When the synthetic medium was buffered at pH 8.5, colonies on its surface were found to be similar to those on beef-extract peptone agar. Furthermore, when ammonium citrate agar plates with red, pink and white colonies were flooded with alkali, all of the colonies became orange-red and could be differentiated only with difficulty. Similarly, when an alkaline beef-extract peptone agar plate with orange-red colonies was flooded with acid, the colonies immediately turned appropriate shades of pink and white. It was evident, therefore, that the pigmentation of colonies grown on beef-extract peptone agar varied as did that

[2] Ridgway color charts.

of those grown on the synthetic ammonium citrate medium, but the differences were conspicuous only on the latter. . It may be noted that in crowded ammonium citrate agar plates the development of an alkaline reaction and orange colonies has frequently been noted after three or more days of incubation.

In order to investigate the stability of each of the five types noted on the synthetic medium, representative dark red, light red, bright pink, pale pink and white colonies were picked to 10 ml. sterile water blanks which were shaken until even suspensions were obtained. One ml. aliquots of 1:1,000,000, 1:100,000 and 1:10,000 dilutions of these suspensions were

TABLE 1

Analysis of colonies obtained by plating S. marcescens #274 on buffered ammonium citrate glycerol agar

COLOR OF PARENT COLONY	NUMBER OF COLONIES SELECTED	AVERAGE NUMBER OF DAUGHTER COLONIES COUNTED	PER CENT OF DAUGHTER COLONIES WHICH WERE				
			Dark red	Light red	Bright pink	Pale pink	White
Dark red.... ...	10	497	98 ± 1	+	2 ± 1	0	0
Light red...	5	382	60 ± 5	+	40 ± 5	0	0
Bright pink. . . .	10	508	41 ± 2	+	58 ± 2	+	0
Pale pink I	7	637	19 ± 4	+	53 ± 3	28 ± 4	0
Pale pink II. .	3	578	14 ± 3	+	58 ± 8		27 ± 11
White..	5	512	5 ± 2	+	31 ± 3		63 ± 6

+ indicates less than 1 per cent.

pipetted into sterile Petri plates, and pour plates were made by adding about 12 ml. of the ammonium citrate agar (45-48°C.). After incubating at 30°C. for three days the plates were examined and the surface colonies were counted and classified as to color. Plates containing between 50 and 400 surface colonies were used. They were viewed by transmitted blue light.

The results of this analysis of the variant colonies are presented in table 1. The dark red colonies were found to be the most stable. Approximately 98 per cent of the viable cells from three-day-old dark red colonies produced daughter colonies which resembled the parents in color; such cells are referred to as dark red cells, although no observations have been made on the colors

of individual cells.   The dark red colonies also contained about 2 per cent of cells which produced bright pink colonies.   These cells, which must have arisen as variants from the dark red cells, were classified as bright pink cells.   No pale pink or white variant colonies were noted, but a few light red colonies were seen.

Very few of the daughter colonies on the plates made from the light red colonies were light red.   About 60 per cent of the cells in the colonies proved to be of the dark red type and the remaining 40 per cent were bright pinks.   It was decided, therefore, that the light red colonies probably resulted from fortuitous mixtures of dark red and bright pink cells, rather than from cells of a specific light red type; if such existed, they were so unstable that they could be ignored.

The bright pink colonies were found to contain about 58 per cent of bright pink colony-forming cells and 42 per cent of variants of the dark red type.   From this it was judged that the bright pink cells were quite unstable and frequently reverted to dark red.   Occasional pale pink colonies were also noted on these plates, and these were thought to arise from variant cells of a third, or pale pink, type.

Ten pale pink colonies were picked from the original plates. Their analysis revealed that they belonged to two different groups.   Seven of the colonies gave daughter colonies of three types: dark red, bright pink and pale pink.   The other three gave dark red, white and intermediate pink colonies, which varied from bright pink to pale pink.   The pale pink colonies of these two groups were thought to come from two types of cells.   Those which gave rise to colonies of the first group were called pale pink cells, although it was realized that they may well have been white in color but so unstable that the resulting colonies always contained enough pigmented variants to give them a pink color.   The origin of the pink colonies of the second group was not at all clear, as very few of the daughter colonies were of the same shade of pink as the parents.   It seemed not unlikely that they had arisen from cells of a fourth, so called white type which produced enough pigmented variants to color the colonies.

The analysis of the white colonies from the original plating of the stock strain showed that they were made up of approximately 68 per cent of cells of the white type which produced colonies that were white or very pale pink, 31 per cent of variants which gave rise to pink colonies most of which were bright pink, and 5 per cent of variants of the dark red type. The white colonies appeared to be closely related to the pale pinks of group II noted above.

From these results it was apparent that colonies of four of the types which were studied, i.e., the dark reds, bright pinks, pale pinks (I) and whites contained an appreciable number of cells that were capable of reproducing the parent type, but that none of these was stable, and that variations could proceed in both directions. In order to test these observations further, colonies of each of these four colors were selected from plates of different series and were replated on synthetic agar. Thus, dark red colonies were picked from platings of dark red, bright pink, pale pink and white colonies. By intentionally selecting the darkest and lightest representatives of each color type, where any differences were evident, it was possible to investigate more completely the limits of variation of the colonies within each type.

Representative results from platings of individual colonies are presented in tables 2, 3, 4 and 5. All of the dark red colonies, whatever their source, proved to be very similar to those analyzed in the first experiment; more than 94 per cent of the cells from each of these colonies were dark red and the variants were all of the bright pink type. The bright pink colonies, including even the darker pinks from the white series, again were found to be composed almost entirely of cells of the dark red and bright pink types. The differences in shade within the type could be correlated in each case with the relative number of dark red variants in the colony, indicating that, although the bright pink cells may have differed somewhat in stability, they did not differ appreciably in color or in the shade of red variant which they produced. In the case of the pale pink colonies also the differences in shade within the type could be correlated with the compositions of the colonies as revealed by plating. The pale

TABLE 2

Analysis of dark red colonies from various sources

SERIES FROM WHICH PARENT COLONY WAS SELECTED	NUMBER OF DAUGHTER COLONIES COUNTED	PER CENT OF SURFACE COLONIES WHICH WERE			
		Dark red	Bright pink	Pale pink	White
Dark red . .. {	501	98	2	0	0
	562	99	1	0	0
	357	96	4	0	0
Bright pink . ...{	428	98	2	0	0
	614	98	2	0	0
	395	97	3	0	0
Pale pink. ....{	452	97	3	0	0
	412	99	1	0	0
	510	97	3	0	0
White.. .. ..{	416	97	3	0	0
	579	99	1	0	0
	327	96	4	0	0

TABLE 3

Analysis of bright pink colonies from various sources

SERIES FROM WHICH PARENT COLONY WAS SELECTED	SHADE OF PARENT COLONY	NUMBER OF DAUGHTER COLONIES COUNTED	PER CENT OF DAUGHTER COLONIES WHICH WERE			
			Dark red	Bright pink	Pale pink	White
Dark red .. . . {	Dark	1,090	42	58	0	0
	Medium	310	31	69	+	0
	Light	527	27	73	0	0
Bright pink . {	Dark	237	54	46	0	0
	Medium	658	33	67	0	0
	Light	476	22	78	+	0
Pale pink.. . . . {	Dark	595	42	58	0	0
	Medium	388	34	65	1	0
	Light	221	23	77	0	0
White.. .... {	Dark	329	41	59	+	0
	Medium	256	31	69	0	0
	Light	416	8	91	0	1

+ indicates less than 1 per cent.

pink colonies which had arisen as new variants from bright pink cells, and those from the pale pink group I series contained no cells of the white type, whereas those from the white series contained an appreciable number.　The white colonies were similar

TABLE 4

Analysis of pale pink colonies from various sources

SERIES FROM WHICH PARENT COLONY WAS SELECTED	SHADE OF PARENT COLONY	NUMBER OF DAUGHTER COLONIES COUNTED	PER CENT OF DAUGHTER COLONIES WHICH WERE			
			Dark red	Bright pink	Pale pink	White
Bright pink..........	Dark	277	30	58	12	0
	Medium	388	24	41	35	0
	Light	365	6	53	41	0
Pale pink　.. 　.....	Dark	258	28	53	19	0
	Medium	358	19	50	30	1
	Light	380	13	60	27	0
White	Dark	275	21	(72)	(2)	5
	Medium	401	16	(37)	(4)	43
	Light	274	6	(53)	(3)	36

( ) indicates rough estimate.

TABLE 5

Analysis of white colonies from various sources

SERIES FROM WHICH PARENT COLONY WAS SELECTED	NUMBER OF DAUGHTER COLONIES COUNTED	PER CENT OF DAUGHTER COLONIES WHICH WERE			
		Dark red	Bright pink	Pale pink	White
White .. 　..............	608	2	(15)	(4)	79
	649	3	(12)	(2)	83
	418	5	(23)	(3)	69

( ) indicates a rough estimate.

to those previously studied and gave a majority of colonies of the white type together with pink and red variants.

The results from these two experiments indicate that *Serratia marcescens* #274, when plated on the synthetic ammonium citrate agar, produces colonies of four principal color types, and that, in spite of minor variations in shade, the different colonies

can be classified as to type with considerable accuracy. Each of these colonies contains an appreciable number of cells which produce daughter colonies of the parent type, but all of the cells are so unstable that three-day-old colonies invariably contain numerous variant cells of one or more of the other color types.

An attempt has been made to stabilize substrains of each of the four principal color types by selective replating. The following procedure has been found to be convenient. A selected colony is picked with a large loop to a 10 ml. water blank; after shaking, a second loopful is carried to a second dilution tube, and from this 0.1, 0.2 and 0.5 ml. are pipetted to Petri plates to which standard agar (at 45–48°C.) is then added. The plates generally contain a good distribution of colonies, from which further selection can be made with the aid of a color chart. A record is kept of the per cent of colonies of each type observed at each transfer. Selective platings have been carried on, in some cases for as long as six months, but in no instance has the stability of any one of the four color types been modified by this procedure. The compositions of the final dark red, bright pink and pale pink and white colonies was strictly comparable with those originally observed and recorded in table 1. This is particularly interesting in view of the fact that relatively stable pink and white strains have been occasionally encountered in the course of aging experiments. Stable dark red strains have not yet been found.

It is not yet known whether qualitative or quantitative differences are responsible for the observed variations in colony color, but preliminary determinations have demonstrated the presence of more than one pigment in ether-alcohol extracts of cells from dark red colonies. When a petroleum ether solution of the total pigments from these cells was run through an aluminum oxide adsorption column one pigment passed through rapidly as a red band, while a second was retained as a purple band at the top of the column. The first gave solutions which were yellow in the presence of alkalies, but bright red upon acidification. This pigment appeared to be the prodigiosin studied by Wrede (1934). The second pigment gave solutions which were red in alkalies but dark purple in acids. These results, when considered in

conjunction with those obtained by flooding the colonies with acids and alkalies, suggested that only the dark red colonies contained appreciable amounts of the purple pigment; but this has not yet been confirmed by chemical analysis.

DISCUSSION AND CONCLUSIONS

When a stock culture *Serratia marcescens* strain #274 was plated on a well-buffered medium such as the ammonium citrate glycerol medium used in these studies, colonies of distinctly different colors were produced. Upon replating it was found that all of these colonies gave rise to daughter colonies of more than one color, but that four types, designated as dark reds, bright pinks, pale pinks and whites, respectively, produced a large number of daughter colonies of the parent type. Although no one of these types proved to be entirely stable the data indicated that each probably arose from a specific type of cell, and therefore that there were four principal types of variant cells. This conclusion was based on the fact that each type of colony, although mixed, was found to contain a characteristic distribution of variant cells as evidenced by the distribution of colony types on plates made from suspensions of individual colonies. This uniformity in the composition of the colonies of each color type indicated they had probably arisen from similar cells and that the cells were characterized not only by their pigment but also by their variability; each type of cell appeared to produce variants in a regular and reproducible manner. Thus, the dark red cells gave off variants at such a rate that three-day-old dark red colonies repeatedly contained between 1 and 6 per cent of bright pink variant cells. Similarly, the bright pink type cells, which were more unstable, produced three-day-old bright pink colonies containing approximately 60 per cent bright pink cells, 40 per cent dark red variant cells and occasional pale pinks. The pale pink type cells gave rise to bright pink variants at an even faster rate, and these in turn gave rise to dark reds. The white type cells were somewhat more stable, but they also produced variants at characteristic rates, as was shown by the uniform distribution of colony types which appeared when white

colonies were replated. The tendency to produce specific variants at constant rates was maintained by each of the four principal color types, in spite of selective replatings in which an attempt was made to stabilize them. Because the production of variants does proceed with such regularity it was practical to characterize cells by the colors of the colonies which they produced, even when these colonies were known to contain more than one type of cell.

SUMMARY

Serratia marcescens #274 when grown on a well-buffered ammonium citrate glycerol medium produced colonies of four principal color types: dark red, bright pink, pale pink and white.

Each of these colonies was thought to arise from a specific type of cell.

When the colonies were replated they invariably gave rise to daughter colonies of one or more variant types, in addition to those of the parent type, indicating that none of the postulated cell types was entirely stable.

The production of variants by each cell type proceeded in such a regular and reproducible manner that plates made from three-day-old colonies of a given color always contained very similar distributions of colonies of the different color types.

The composition of the colonies of each type, as revealed by plating on the synthetic medium, was not affected by selective replatings carried on over a period of six months.

Preliminary analyses of the pigments in cells from dark red colonies demonstrated the presence of two major components, but it is not yet known whether either of these is lacking in the pink cells.

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THE PRODUCTION OF STABLE POPULATIONS OF COLOR VARIANTS OF SERRATIA MARCESCENS #274 IN RAPIDLY GROWING CULTURES

MARY I. BUNTING

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The evidence from the studies reported in a preceding paper (Bunting, 1940) indicated that *Serratia marcescens* #274 yields color variants of four principal types, and that cells of these four types may be recognized by the colors of the colonies which they produce when plated on a well-buffered ammonium citrate glycerol medium. The colonies were described as dark red, bright pink, pale pink or white. When suspensions from individual colonies were plated on the synthetic medium it was found that the colonies invariably contained cells of variant types as well as those of the parent type; but, although all of the cells were shown to be quite unstable, variants arose in such a regular manner that colonies of each color type were found to contain almost equal numbers of variant cells. The experiments reported in this paper were designed to study the rates at which variants were produced by cells of different color types. It was thought that if cultures could be grown for long periods under relatively uniform conditions it might be possible to calculate the rates of variation from data showing the changes in the relative numbers of cells of each color type, providing the rates of multiplication of the cells of each color type could be ascertained.

The medium which was chosen for these studies was the ammonium citrate broth described in the preceding paper.[1] It was tubed daily in 10 ml. amounts in 1 inch test tubes, which were

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[1] This broth contained per liter of distilled water: ammonium citrate 5.0 grams; glycerol 5.0 grams; $K_2HPO_4 \cdot 3H_2O$ 10.0 grams, $MgSO_4$ 0.5 gram; NaCl 0.5 gram; ferric ammonium citrate 0.05 gram.

plugged, autoclaved, and stored at 30°C. The rate of cell multiplication was used as an index to the physiological uniformity of the medium, and transfers were made as frequently as was necessary to keep the cultures growing at a constant rate. Tubes were routinely inoculated with about 10,000 cells per milliliter and were incubated at 30°C. until their turbidities indicated the presence of about 10,000,000 cells per milliliter; transfers were then made to fresh tubes, using 1 ml. of a 1:100 dilution from the turbid cultures. By transferring cultures in this manner at approximately 12-hour intervals it was possible to keep them growing logarithmically over long periods of time, and from the records of the number of transfers made over any time interval it was an easy matter to calculate the rates of cell multiplication for that interval. The relative number of cells of each color type in the fast-growing cultures was found by plating samples and observing the relative number of surface colonies of each color on three-day-old synthetic agar plates.

In the first experiment to be reported, duplicate tubes were inoculated with suspensions of cells from dark red, light red and bright pink colonies. The six series of tubes were observed at frequent intervals and were transferred in the manner described above. At two and three day intervals samples were taken for plating. Previous work had shown that when suspensions from colonies of these three colors were replated, 99 per cent of the daughter colonies were either dark red or bright pink, and that the parent colonies differed only in the proportions of cells of these two types which they contained. It was desired to find out whether these proportions would change on prolonged cultivation under uniform conditions, and, if they did change, to what extent the changes could be ascribed to differences in growth rates of cells of the different color types, or to what extent they were due to the production of variants by these cells.

The results are presented in table 1 and graph 1. There were no appreciable differences in the rates at which the tubes became turbid, either at the beginning or at the end of the experiment. The generation times of the different series, as calculated from the record of transfers, were approximately 65 minutes in each

instance and were constant throughout the experiment.   It was concluded, therefore, that there was no significant difference between the rates of cell multiplication of dark red and bright pink type cells under these conditions.   In spite of this, however, there were very definite shifts in the relative numbers of cells of the two types in the fast-growing cultures.   In each case the proportion of cells of the dark red type increased as the experiment proceeded.   Series 1 and 2 were inoculated from the dark

TABLE 1

Rate of increase in the per cent of dark red cells in fast-growing cultures, inoculated with mixtures of dark red and bright pink type cells

	SERIES NUMBER					
	1	2	3	4	5	6
	Color of colony used as inoculum					
	Dark red	Dark red	Light red	Light red	Bright pink	Bright pink
Generation Time in minutes:						
First 2 days...	65	65	65	65	65	62
Entire 12 days	64	64	65	65	64	64
Per cent of dark red colony forming cells in:						
Original inoculum.. .	93	93	66	66	23	23
2-day cultures..	96	95	79	80	45	44
4-day cultures...	96	94	86	83	62	61
7-day cultures	98	98	88	89	78	74
10-day cultures..	96	98	91	88	84	85
12-day cultures.. . .	97	98	92	94	87	88

red colony which contained 93 per cent dark red type cells and 7 per cent bright pinks; after 7 days the per cent of dark reds had increased to about 98 per cent where it remained for the following four days.   The per cent of dark red colony-forming cells in series 3 and 4 inoculated from the light red colony rose in 12 days from 66 per cent to between 92 and 94 per cent, and in series 5 and 6 inoculated from the bright pink colony there was a shift from 23 per cent dark reds and 77 per cent bright pinks to ap-

proximately 87 per cent dark reds and 13 per cent bright pinks. It may be seen from graph 1 that in each case the increase in the per cent of dark red colony-forming cells proceeded at a constantly decreasing rate, and seemed to be directly related to the number of bright pink type cells which were present. The populations appeared to come to equilibrium with about 97 per cent dark reds and 3 per cent bright pinks. This was confirmed
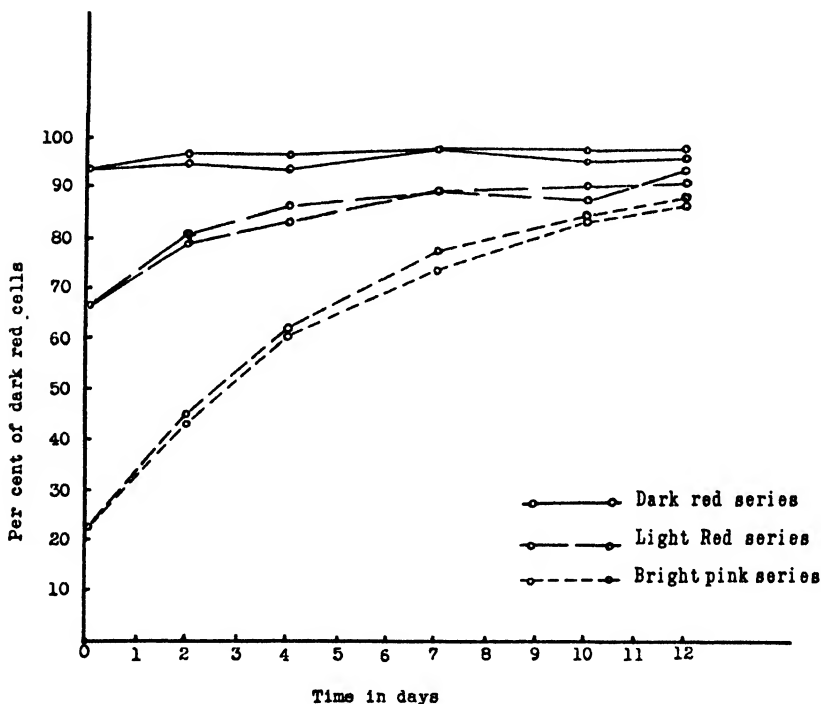
GRAPH 1

by later experiments in which cultures were maintained by rapid transfer for 30 days.

Since these shifts in population could not be accounted for by any differences in growth rates, it was assumed that they were entirely due to differences in rates of variation. It was known from previous plating experiments that dark red type cells produce an appreciable number of variants of the bright pink

type and that these in turn frequently revert to the dark red type. If it is assumed that the rates of variation of these two cell types are constant, then, in growing cultures where both types are multiplying at the same rate, the populations should eventually come to equilibrium. Thus, if 97 dark red cells produce 1 bright pink cell in the same length of time that 3 bright pinks produce one dark red, the population will come to equilibrium with 97 per cent dark reds and 3 per cent bright pinks. Such an equilibrium population may be described by a mass law equation such as:

$$(1) \qquad \frac{R\infty}{P\infty} = K = \frac{Pr}{Rp}$$

where $R\infty$ and $P\infty$ are the per cents of dark red and bright pink type cells at equilibrium, Rp is the per cent of dark red cells that form bright pink variants in a given time interval and Pr is the per cent of bright pink type cells that form dark reds in the same interval. If $R\infty = 97$ and $P\infty = 3$ as in the first experiment, then

$$K = \frac{97}{3} = 32.3$$

and

$$Pr = 32.3\ Rp$$

which indicates that the bright pinks produce dark red variants about 32 times as fast as the dark red produce bright pinks.

Furthermore, the changes in the relative number of cells of each color type in a standard culture that is not at equilibrium may be described by equation (2):

$$(2) \qquad R_t = Ro\ (1\infty - Rp) + Po\ (Pr)$$

where Ro and Po are the per cents of red and pink cells in the culture at any given moment, R_t is the per cent of reds present after a time interval t, and Rp and Pr are again the per cents of red and pink cells that form variants of the opposite type in the interval t. Using this equation and substituting $Pr = 32$ Rp, $Ro = 45$, $Po = 55$ and $R_t = 65$ (table 1, series 5, 2 to 4

days), Rp was calculated to be 0.0099, indicating that about 0.01 per cent of the dark red cells formed bright pink variants in the interval from 2 to 4 days. From equation I it followed that Pr = 0.32, and therefore that in the same two-day interval 0.32 per cent of the bright pink cells produced dark red variants. Simi-

TABLE 2

Theoretical changes in the relative number of dark red cells in fast-growing cultures inoculated with suspensions of bright pink and dark cells

TIME	PER CENT OF DARK RED CELLS[*]	
	Inoculum of bright pink cells	Inoculum of dark red cells
days		
0	0	100
2	32.0	99.0
4	53.5	98.3
6	67.9	97.7
8	77.5	97.4
10	83.9	97.2
12	88.3	97.1
14	91.2	97.0
16	93.1	97.0
18	94.4	97.0
20	95.3	97.0
22	95.9	97.0
24	96.2	97.0
26	96.4	97.0
28	96.6	97.0
30	96.8	97.0
32	96.9	97.0
34	97.0	97.0
36	97.0	97.0

* These figures are calculated from equation 2 by substituting Rp 0.01, Pr 0.32 ($t = 2$ days).

lar values were obtained when data from other two-day intervals were used.

By substituting $Rp = 0.01$ and $Pr = 0.32$ in equation 2 it was possible to calculate the theoretical changes in the numbers of dark red and bright pink cells in similar cultures inoculated with suspensions of dark red or bright pink cells. The results of these calculations are presented in table 2 and plotted as

smooth curves in graph 2.   Theoretically, by adjusting the origin in a suitable manner these curves can be used for predicting changes in any population of dark red and bright pink cells that are growing under similar conditions, provided that the two color types continue to multiply at equal rates and that their rates of variation remain constant.   When the data from table 1 were plotted in this manner, they were found to fit the curve rather well as may be seen from graph 2.   However, other substrains have been found which come to equilibrium at slightly different rates, so that at the present time too much emphasis
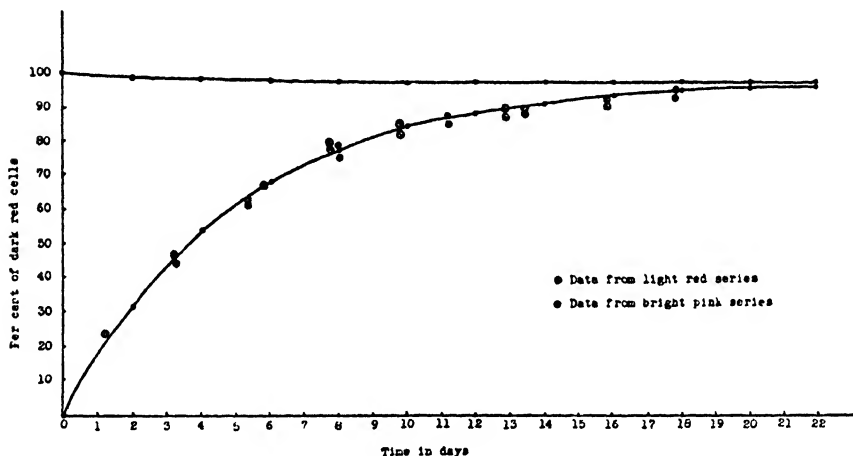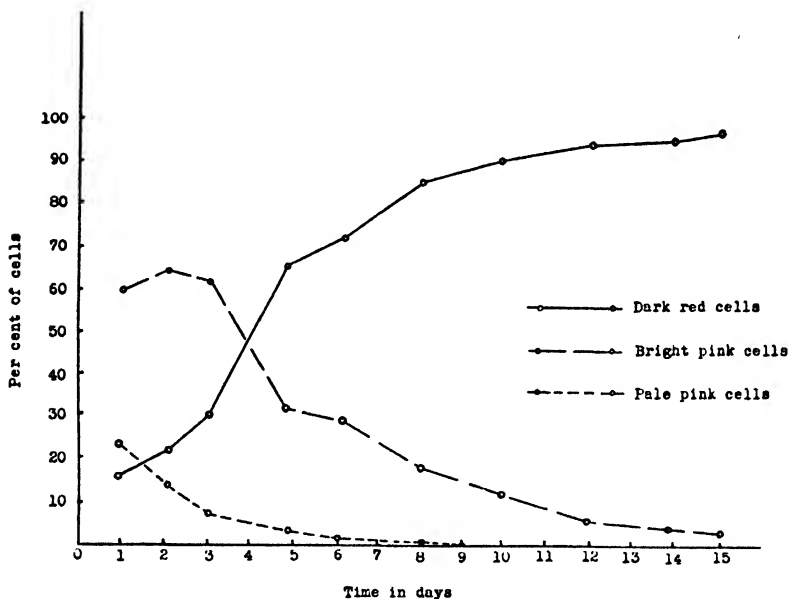

GRAPH 2

should not be placed on the specific values which have been obtained for the rates of variation of the dark red and bright pink type cells.   This is to be anticipated, particularly, when one considers how seriously these values are affected by any error in the determination of K from the equilibrium populations.   It should be noted also that in some cases cultures have behaved in an atypical fashion during their first few days of logarithmic growth.   Since February 1939 when the experimental results reported in table 1 were obtained, 18 mixtures of dark red and bright pink cells have been grown and studied and in every instance the populations have reached equilibrium with between

96 and 98 per cent of dark red type cells. A certain amount of variation has been noted, however, both in the rate of cell multiplication and in the rate of attaining equilibrium of cultures from different substrains.

Experiments have also been performed in which cultures inoculated with cells from pale pink and white colonies have been grown in a similar manner. Typical results are given in graphs 3 and 4. Previous studies have shown that pale pink colonies

Per cent of cells

Dark red cells
Bright pink cells
Pale pink cells

Time in days

GRAPH 3

are generally composed of cells which give rise to dark red, bright pink and pale pink daughter colonies. The changes in the relative numbers of each of these cell types during logarithmic growth in the synthetic broth are shown in graph 3. The pale pink type cells fell steadily from 24 per cent at 1 day to less than 1 per cent at 7 days, while the bright pink type cells rose from 60 per cent at 1 day to 64 per cent at 2 days and then fell, at a constantly decreasing rate, to about 4 per cent at the end of 15 days; in the meantime the dark red type cells rose from 16 to

96 per cent.   Similar changes were seen in the series (graph 4) inoculated from white colonies.   These colonies are composed of cells which give dark red, pink (mostly bright pink) and white daughter colonies.   The number of white type cells fell rapidly from 68 per cent at 1 day to less than 1 per cent at the end of the experiment.   The bright pink type cells rose from 26 per cent at 1 day to 56 per cent at 4 days and then fell to about 7 per cent at 15 days.   The dark reds rose from 7 per cent to 93 per cent in the same length of time.   In both of these series,
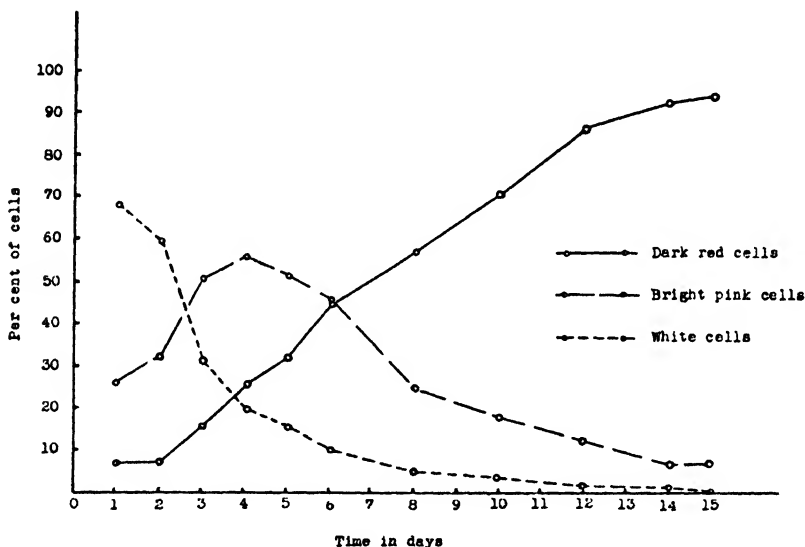
GRAPH 4

which were inoculated from colonies obtained from a relatively slow-growing substrain, the generation times were about 71 minutes and were constant throughout the experimental period. There was no evidence, therefore, that there were any differences in the growth rates of any of these color variants, and, again, the changes in the fast-growing populations were ascribed to differences in the stability of the different cell types.   The initial temporary increase in bright pink cells in both series indicated that most if not all of the variants produced by the pale pink and

white type cells were of the bright pink type, and that these were produced more rapidly than they gave rise to dark red variants. As yet no attempt has been made to calculate the actual rate of production of bright pink variants from pale pink and white type cells.

A study is now being made of the effects of various environmental conditions on the behavior of fast-growing cultures of *Serratia marcescens*. The production of stable populations containing approximately 97 per cent of dark red cells and 3 per cent of bright pink proceeds as readily in ammonium citrate broth buffered at either pH 6.0 or 8.5 as at pH 7.0. No change was produced by poising the medium at a low oxidation-reduction potential with thioglycollic acid. The addition of either glucose or peptone to the synthetic medium increased the rate of cell multiplication in fast-growing cultures but did not affect the rate at which these cultures attained equilibrium. Very different, however, were the preliminary results obtained at different temperatures. In this case the rates of increase of dark red cells and the final equilibrium populations as well as the rates of cell multiplication were affected. Thus, in one experiment, populations grown at 18°C. had a generation time of 156 minutes and an equilibrium population of 99 per cent dark reds and 1 per cent bright pink, whereas controls at 30°C. had a generation time of 71 minutes and an equilibrium population with 97 per cent dark red and 3 per cent bright pinks, and a third series at 37°C. had a generation time of 62 minutes and an equilibrium population of 88 per cent dark reds and 12 per cent bright pinks. The curves at 37°C. were not smooth; during the early stages of growth when the per cent of dark red cells was low, these cells increased faster at 37°C. than in control cultures at 30°C. but after the dark red cells had increased to about 70 per cent there was a definite break and the subsequent increase in these cells was very slow. Further studies are being made of this phenomenon.

DISCUSSION

When cultures of *Serratia marcescens* #274 were plated and replated as reported in the previous paper, they proved to be very

unstable. Dark red, bright pink, pale pink and white colonies were produced but none of these "bred true" and it was not possible to stabilize them by selective platings. Variations were so frequent and at the same time so conspicuous that they presented somewhat perplexing taxonomic problems. However, the procedure adopted in the experiments reported in this paper where cultures were grown logarithmically under standardized conditions brought out essential relationships between the different color variants. It has been found that under these standard conditions of growth, all of the variant color types eventually produced stable populations with about 97 per cent of dark red-colony forming cells and 3 per cent of bright pinks. This procedure has been successful in stabilizing the cultures because, rather than attempting to ignore the inherent variability of the cells, it has taken advantage of the fact that specific variations occur with characteristic frequencies and can be depended upon to control the cellular composition of cultures when other interfering forces are non-operative. The production of unstable cells of different color types· by *Serratia marcescens* ⚹274 is one of its most characteristic properties, and any method which enables one to measure this tendency provides a valuable means of describing the organism. It will be interesting to see how many other strains of *Serratia* will behave similarly to strain ⚹274 when grown under similar conditions.

To what extent procedures of this type will prove useful in stabilizing cultures of other species remains to be seen. Ordinarily one must expect that a given environment will favor the growth of one variant over another, and that therefore the composition of the resulting population will depend on survival values in the ordinary Darwinian sense, as well as on the rates of variation of the respective variants. In the case of the particular variants which were studied in these experiments the situation was simplified because all of the variants happened to grow at an equal rate under the conditions imposed. It was therefore particularly easy to observe that specific variations were proceding at constant rates in accordance with the original assumptions. The observed rates of variations were considered to be expressions of the probability of the occurrence of the specific

intracellular events which are favorable to the production of variants.

The nature of the intracellular events which result in the production of variants is unknown. They may be gene mutations or they may be phenomena of quite a different sort. In any case the methods outlined in this paper can easily be modified to permit a study of the effect of various environmental factors on the rate of variation; such studies are now in progress. Information along these lines may or may not prove helpful in deciding whether bacterial variations are analogous to mutation in higher plants and in animals; it should contribute to our knowledge of bacterial variation.

SUMMARY

When cultures of *Serratia marcescens* #274 inoculated from variant dark red, bright pink, pale pink and white colonies were grown under carefully controlled conditions all of them eventually came to equilibrium with about 97 per cent of dark red type cells and 3 per cent of bright pink type cells. There was no indication that any one of these variant types multiplied at a faster rate than any of the others under the experimental conditions, and therefore it was concluded that the observed changes in the compositions of the fast-growing cultures were due entirely to the relatively greater instabilities of the less highly pigmented types. Calculations based on one experiment with cultures of dark red and bright pink cells indicated that about 0.01 per cent of the dark red cells of this particular substrain produced bright pink variants every two days, whereas in the same interval 0.32 per cent of the bright pinks produced dark red variants. The production of paler variants by either of these types was evidently very much less frequent. No figures are yet available for the rates of variation of pale pink and white type cells but the data indicate that these types are even more unstable than the bright pinks and that the majority of variants which they produce are of the bright pink type.

Preliminary studies have given no indication that the hydrogen ion concentration or the oxidation-reduction potential or the

chemical composition of the medium affects the rate of production of variants, but there is evidence that temperature does influence the stability of different color variants.

REFERENCE

BUNTING, M. I.   1940   A description of some color variants produced by *serratia marcescens*, Strain 274.   J. Bact., **40**, 57–68.

AN ECOLOGICAL STUDY OF THE COLIFORM BACTERIA

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Previous papers (Stuart, Griffin, and Baker 1938, Stuart, Wheeler, and Griffin 1938) presented the results of an extensive study of the types of coliform organisms encountered in milk and in the immediate environment of milk production. The various reactions of the strains in the "IMViC" differential media (indole, methyl-red, Voges-Proskauer, citrate, and cellobiose) led to the schematic classification shown in figure 1. Evidence validating this arrangement was obtained from "shifts" in reaction of certain unstable strains and statistical analysis of correlation among the reactions of the entire population. This analysis showed that there was no contraindication to adoption of the scheme as a basis for the more detailed classification of the coliform group.

Although such a classification is theoretically adequate, it must be shown that separation of the *Aerobacter*, intermediate, and *Escherichia* sections of the group by such means will meet the demands of the practical worker in regard to sanitary significance. This paper presents the results of an ecological study made with this end in view.

The methods, essentially those previously described (Stuart, Griffin, and Baker 1938), involved preliminary plating on eosin methylene-blue agar (Stuart, Wheeler and Griffin 1938) followed by transfer of representative numbers of typical and suspected coliform colonies to lactose broth and the IMViC differential media. The only modification used was preliminary enrichment in lactose broth in those instances where coliform organisms were too few in number to permit direct plating. When this was done,

duplicate strains from a single lactose tube were eliminated from the final tabulation.

A total of 6577 strains from milk, soils, grains, water, and feces were classified by this technique. The percentage distribution by types is given in table 1. The results obtained by Stuart, Griffin, and Baker (1938) are included so that the distribution of all cultures studied in this laboratory may be

RELATIONSHIPS OF COLIFORM ORGANISMS

REACTIONS
INDOL METHYL-RED VOGES-PROSKAUER CITRATE CELLOBIOSE

FIG. 1
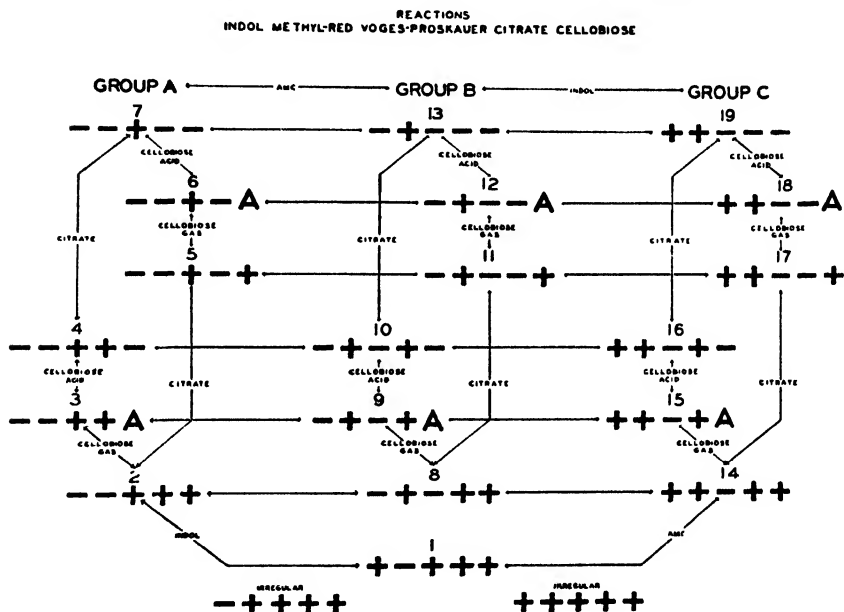
compared (table 2) with that given by previous investigators. The coliform sections in this tabulation are based on the use of the Voges-Proskauer and citrate reactions since this was the basis used in most of the studies. The results from the present investigation were reclassified on this basis for purposes of comparison as were those previous studies, similarly based on the IMViC system. Such reclassification has no significant effect on the distribution. Some recent studies of the coliform group have been omitted since they were directed primarily at the intermediate section and made no pretense of statistical sampling.

TABLE 1

Percentage distribution of coliform organisms from various sources

SOURCE	NUMBER	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	irr.
Milk*	1358	7.8	50.8	0.0	0.0	0.7	0.3	1.3	14.7	4.9	2.1	0.0	0.0	0.5	2.1	0.0	0.0	2.6	0.0	11.3	0.8
Water	1366	6.8	25.2	0.5	0.6	0.7	0.2	0.1	3.2	3.9	0.2	0.3	0.2	1.7	2.8	0.4	0.6	1.6	0.2	40.0	2.1
Soils	617	9.8	62.4	0.3	0.2	2.2	1.3	0.5	3.4	1.3	0.3	0.5	0.0	0.5	0.7	0.8	0.2	2.2	0.2	12.0	0.8
Grains	512	16.8	52.5	1.2	0.4	0.4	0.8	0.0	4.1	2.1	0.4	13.5	0.2	0.0	0.6	0.0	0.0	2.1	0.2	3.9	
Dust and Hides*	1228	6.8	47.3	0.6	0.0	0.1	0.1	0.0	4.0	3.6	0.4	0.0	0.6	1.8	2.3	0.0	0.0	0.9	0.2	31.3	
Feces, Human	1113	1.8	9.4	0.0	0.0	0.0	0.0	0.0	2.4	2.5	2.5	0.0	0.1	2.0	1.1	0.4	1.2	2.7	0.4	73.3	
Feces, Cows*	383	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	4.4	0.0	0.0	0.0	0.0	0.6	95.6	
Total	6577	6.9	38.2	0.3	0.1	0.6	0.3	0.3	5.5	3.3	0.8	1.6	0.2	1.4	1.8	0.2	0.3	1.8	0.2	35.6	0.4

Group A = 39.8   Group B = 12.7   Group C = 40.2

* From: Stuart, Griffin, and Baker 1938.

TABLE 2

Percentage distribution of coliform organisms from various sources as found by different authors

AUTHOR	MILK Number	Esch.	Aero.	Int.	Irr.	WATER Number	Esch.	Aero.	Int.	Irr.	SOIL Number	Esch.	Aero.	Int.	Irr.	GRAINS Number	Esch.	Aero.	Int.	Irr.	FECES Number	Esch.	Aero.	Int.	Irr.
Bardsley (1934)	324	53.1	22.5	24.4	0.0	4297	70.5	13.8	15.4	0.3	152	30.9	2.6	66.5	0.0						327	78.6	12.2	9.2	0.0
Bartram and Black (1937)																					423	92.6	3.8	2.4	1.2
Burke-Gaffney (1932)						1423	25.7	53.4	15.2	5.7	244	54.5	26.6	12.7	6.2						357	89.6	0.6	9.8	0.0
Kline (1935)	266	35.3	54.9	9.8	0.0	317	31.5	28.4	40.1	0.0	30	76.6	7.0	16.7	0.0	75	4.0	90.7	5.3	0.0	118	89.8	7.6	2.6	0.0
Koser (1924)											72	2.8	65.3	31.9	0.0						79	96.2	3.8	0.0	0.0
Koser (1926)											162	11.1	71.0	17.9	0.0						103	97.1	0.0	2.9	0.0
Koser and Saunders (1932)																									
Oeser (1937)						2093	38.4	31.9	28.9	0.8															
Ruchhoft et al. (1931)	164	50.0	34.2	15.2	0.6																486	94.2	2.5	3.3	0.0
Skinner and Brudnoy (1932)																					585	89.1	0.2	10.7	0.0
Yale (1933)	112	61.6	23.2	15.5	0.0																				
Total (previous studies)	866	48.2	34.7	17.0	0.1	8130	52.9	25.9	19.8	1.4	713	31.3	36.6	30.0	2.1	75	4.0	90.7	5.3	0.0	2478	90.0	3.3	6.5	0.2
Present study	1358	16.5	58.6	22.6	2.3	1366	44.0	43.9	11.2	0.9	617	15.2	74.9	5.8	4.1	512	19.9	71.3	7.6	1.2	1496	84.1	8.4	7.5	0.0
Grand total	2224	28.8	49.5	20.3	1.4	9496	51.6	28.6	18.5	1.3	1330	23.8	54.3	18.8	3.1	587	17.9	73.8	7.3	1.0	3974	87.9	5.2	6.8	0.1

85

Considerable caution should be exercised in drawing conclusions from the data of table 2. There is a high degree of variability among the findings of the various authors which may in some instances be due to the small number of strains studied, but which must be due to other factors where the number of strains is sufficiently large to be considered as a statistical sample. The mean values for the entire series are also open to question since in most cases they are heavily weighted by the work of some single author. Significant conclusions can therefore be drawn only if the variability is less than that which would be expected if a series of samples were drawn from a homogeneous population. The chi-square test for homogeneity as applied to the distribution data gives the results shown in table 3. For the sake of simplicity, only the regular sections of the coliform group have been included. To indicate statistical homogeneity chi-square should have the following values for the grand totals: milk and water (df = 8) 15.5, soil (df = 12) 21.0, and feces (df = 16) 26.3. In all instances the total chi-square values are far in excess of these limits; hence the likelihood that any source represents a homogeneous coliform population is extremely remote. Of the individual sections of the coliform group the same holds true with the exception of fecal *Escherichia*. Here $\chi^2 = 11.4$ (df = 8) is well within the significant value of 15.5. Thus homogeneity of *Escherichia* from feces is particularly significant with respect to ecological considerations as will be seen later. With this exception all chi-squares are of such magnitude that it is apparent that even so large a series of cultures can not be depended on to furnish a satisfactory concept of the distribution of the coliform population in general.

These data show the fallacy of drawing conclusions from a simple comparison of percentages of the coliform sections as derived from different sources by different authors. Milk and other prepared food stuffs draw upon the environment under which they are produced for their coliform flora; water and soil will be influenced by the degree of exposure to contamination; and fecal material will vary widely from individual to individual and even geographically. In this latter respect the results ob-

TABLE 3

Values of chi-square for distribution of coliform organisms as found by various investigators

AUTHOR	MILK				WATER				SOILS				FECES			
	Esch.	Aero.	Int.	Total	Esch.	Aero.	Int.	Total	Esch.	Aero.	Int.	Total	Esch.	Aero.	Int.	Total
Bardsley (1934)	63.3	49.1	2.1	114.5	297.9	326.9	22.9	647.7	2.5	77.3	173.3	253.1	3.2	31.1	2.4	36.7
Bartram and Black (1937)																
Burke-Gaffney (1932)	3.4	1.2	15.4	20.0	185.7	309.9	9.0	504.6	104.5	31.2	4.1	139.8	1.7	1.6	12.5	15.8
Kline (1935)					24.7	0.0	78.4	103.1	32.9	13.0	0.1	46.0	0.1	14.8	4.4	19.3
Koser (1924)									13.9	1.1	5.8	20.8	0.1	1.4	3.2	4.7
Koser (1926)									12.0	6.5	0.2	18.7	0.6	0.3	5.5	6.4
Koser and Saunders (1932)									13.0	0.1	21.0	34.1	1.0	5.4	2.4	8.8
Oeser (1937)	24.9	7.9	2.3	35.1												
Ruchhoft et al. (1931)					70.5	8.3	118.4	197.2					2.2	7.0	9.1	18.3
Skinner and Brudnoy (1932)													0.1	28.4	12.6	41.1
Yale (1933)	4.0	16.2	1.7	21.9												
Present study	69.0	25.9	3.8	98.7	15.3	114.0	40.2	169.5	18.3	51.4	54.2	123.9	2.4	28.6	0.8	31.8
Grand total	164.6	100.3	25.3	290.2	594.1	759.1	268.9	1622.1	197.1	180.7	258.6	636.4	11.4	118.7	52.9	182.9

tained by Burke-Gaffney (1932) and Koser (1924, 1926) are interesting. Burke-Gaffney studied two series of fecal cultures, one of tropical, the other of European origin. Koser classified two series presumably from the same geographical locality but spaced two years apart. Statistical analysis of their results shows that of Burke-Gaffney's series only the intermediate section was homogeneous (x^2 = 0.48); the total chi-square being 36.6 (df = 3). In Koser's studies the entire coliform flora was drawn from a homogeneous population (x^2 = 6.9, df = 3). Similar circumstances are encountered in their studies of coliform organisms from soil. Here again Burke-Gaffney's intermediate strains were homogeneous (x^2 = 1.36), although the entire population was not (x^2 = 138.4, df = 3). Koser's studies very nearly indicated homogeneity (x^2 = 10.8, df = 3) for the entire group, although the degree of probability was not as great as was the case with feces.

It is not surprising that cultures from soil should exhibit considerable variation in distribution in the hands of different workers. There is considerable evidence indicating that there is a differential survival rate favoring *Aerobacter* and intermediates over *Escherichia* in fecal material outside the animal body (Ruchhoft *et al.* 1931, Parr 1936) which would have a marked effect on the distribution of the sections depending on the remoteness of contamination. Burke-Gaffney (1932) found significant differences when his strains were segregated on the basis of sanitary surveys of the regions from which they were taken. Our own strains, similarly treated, are distributed as in table 4. Recently contaminated soil was obtained from pasture land, barnyards, and recently fertilized fields. Remotely contaminated soil had been fertilized 18 to 24 months prior to taking the samples. Uncontaminated soil was from a reservoir watershed which had been under rigid control for more than ten years.

The decrease in *Escherichia*, as the opportunity for fecal contamination diminishes, is immediately apparent, as is the relative constancy of *Aerobacter*. Intermediates seem to increase in relative numbers, but this apparent increase has little statistical significance. The chi-square test applied to these data (table 4)

suggests some interesting conclusions. As would be expected, the three types of soil do not present a homogeneous population ($x^2 = 92.2$, df $= 4$) with respect to the entire coliform group nor with respect to *Escherichia* ($x^2 = 72.3$, df $= 2$). *Aerobacter* and intermediates, while not strictly homogeneous, have values of chi-square with probabilities of about 0.01 and 0.04 respectively, the latter being just below the accepted standard of significance. It will be recalled that Burke-Gaffney's geographically separated series also showed homogeneity of the intermediate section. These facts suggest strongly that *Aerobacter* and intermediates constitute the normal coliform flora of the soil, since their occurrence is statistically independent of fecal contamination.

TABLE 4

Distribution of coliform organisms from soil with respect to sanitary conditions

SANITARY CONDITION	ESCH.		AERO.		INT.		TOTAL	
	Number	x^2	Number	x^2	Number	x^2	Number	x^2
Recent contamination. ..	71	45.6	127	6.8	7	2.4	205	54.8
Remote contamination .	19	6.5	173	0.2	21	4.9	213	11.6
No contamination	4	20.2	162	5.0	8	0.6	174	25.8
Total.............	94	72.3	462	12.0	36	7.9	592	92.2

Similarly, with water, Burke-Gaffney (1932) found significant differences in the concentration of *Escherichia* when the results were correlated with the sanitary survey. It is not possible to treat our strains from water on the basis of sanitary surveys, but they can be segregated into two major groups. Two hundred and ninety-two strains were isolated from Rhode Island waters furnished by the State Department of Health. Another group comprising 912 strains were obtained from the Connecticut State Department of Health through the courtesy of Dr. F. L. Mickle. These two groups are compared in table 5. The lack of homogeneity is apparent from the large chi-square for the total population. The same is true of the *Escherichia* and *Aerobacter* sections. The wide discrepancy in this instance is probably due to the fact that the Rhode Island cultures represent strains isolated during

routine control work while those from Connecticut are more circumscribed as to source. Even so, it is notable that the intermediate section is still homogeneous ($\chi^2 = 3.0$). The connotation of these facts will be considered later.

As has been stated, the use of either citrate utilization or indole production in conjunction with the Voges-Proskauer reaction for purposes of classification of members of the coliform group is possible without significantly altering the distribution of the sections as found in various sources. The use of indole production as a criterion will, then, satisfy the requirements of the practical worker, and in addition is more satisfactory from the taxonomic point of view.

TABLE 5

Comparison of two groups of coliform organisms from water

SOURCE	ESCH.		AERO.		INT.		TOTAL	
	Number	χ^2	Number	χ^2	Number	χ^2	Number	χ^2
Conn........	560	26.9	247	29.3	102	0.7	909	56.9
R. I....	35	83.5	213	91.1	44	2.3	292	176.9
Total	595	110.4	460	120.4	146	3.0	1201	233.8

Previous observations on the instability of certain cultures (Stuart, Griffin, and Baker 1938) with respect to the IMViC reactions have been extended to the point where the relationships indicated in figure 1 are strongly confirmed. Figure 2 shows this graphically. The arrows in the chart represent shifts in reaction which have been encountered, and the numbers breaking the arrows indicate the frequency with which the shifts occurred. In no instance does an arrow represent a single observation. The data from which the chart was constructed comprise approximately 1500 observations on 457 strains. Of these, 116 were unstable. This does not imply, however, that 25 percent of all coliform cultures are unstable in their reactions in the IMViC differential media. The cultures selected for study were unusual types (other than 2 or 19) or displayed some anomaly that suggested further observations.

The greatest lack of stability was encountered in types 8 and 9. Nineteen of 99 cultures of these two types oscillated between these types. This fact is perhaps due to the limitations of time imposed on our observations. In routine study the maximum time of observation of cellobiose is 5 days. All of these cultures produced gas in cellobiose provided the time limit was extended sufficiently, although 21 days were required in a few instances.

VARIABILITY OF COLIFORM ORGANISMS
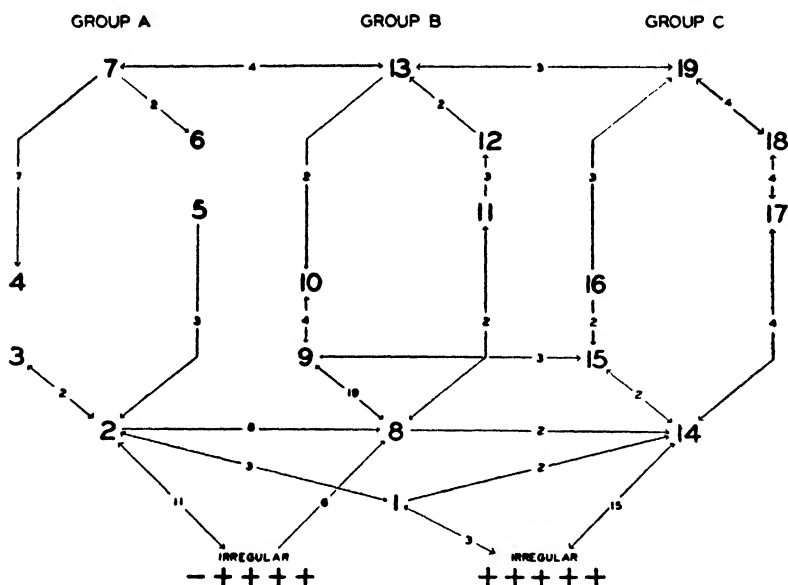
Fig. 2

Stable cultures of type 9, on the other hand, failed to produce gas in cellobiose in 6 weeks. Seventeen and 18 irregular cultures, of the types $(-++++)$ and $(+++++)$ respectively, were unstable. These shifts are independent of incubation temperature, the effect of which will be discussed later. It is significant that only 20 of the 116 shifts observed were from group to group, and of these 8 were between types 2 and 8. Shifts between types 7 and 13 were not spontaneous. They were observed by Bitterlich (1939) in selection experiments.

Another type of variability centers around the Voges-Proskauer reaction. In these instances there was a shift in type due to the V-P reaction being positive when the cultures were incubated at room temperature and negative at incubator temperature. In this manner 12 cultures of type 1 at room temperature were type 14 at 37°C., and 12 of type 2 shifted to type 8. Single instances have been observed of types 3, 5, and 6 shifting to types 9, 11, and 12 respectively under similar conditions. This type of variability is different from that previously described (Stuart, Griffin, and Baker 1938) where irregular cultures became methyl-red negative at room temperature or if the period of incubation before testing was prolonged.

The percentage of irregular cultures listed in table 1 (0.4 per cent) is far lower than would be anticipated from the previous report where 9 per cent were encountered. This is due to the fact that table 1 lists only those cultures that could not be classified by retesting at room temperature. Actually, 88 per cent of the cultures of group A isolated from uncontaminated soil were irregular to some degree, while of the total number of soil cultures 37 per cent were irregular. Thirty-six and 40 per cent of group A cultures from water and grains respectively were irregular at 37°C. From feces, however, only two (0.6 per cent) irregular strains were found at 37°C.

Stuart, Griffin, and Baker (1938) reported approximately 20 per cent of "atypical" cultures which were anomalous in lactose fermentation. It seemed desirable to extend the terminology applied to these cultures; hence we now term as "anaerogenic" those cultures which fail to produce gas from lactose but otherwise appear to belong to the coliform group; such strains as are delayed in fermentation and produce only small amounts of gas are called "microaerogenic"; while that type which produces no gas at 37°C. but a normal amount at room temperature has been designated "pseudomicroaerogenic." Table 6 shows the distribution of these anomalous strains. The number of aberrant cultures from water would probably be higher but for the fact that the water strains were obtained from routine laboratories and hence were presumably normal fermenters. The fact that a

certain number of strains were aberrant on retesting in this laboratory is probably due to the fact that cultures frequently become deficient in ability to attack lactose on cultivation under laboratory conditions. The per cent of microaerogenic strains from soil is probably low. All of the soil cultures isolated regardless of sanitary condition, were used as a basis for computing the percentages, and there are undoubtedly a considerable number of strains of fecal origin present in the series. In one series of soil cultures from an uncontaminated area 97 per cent of all strains were microaerogenic.

In view of Parr's (1934, 1936, 1938a, b) observations on slow lactose fermenters in feces we had anticipated encountering a considerable number in the course of this work. Of all the ab-

TABLE 6

Anomalous lactose fermentation by coliform organisms

SOURCE	ANAEROGENIC		MICROAEROGENIC		PSEUDO-MICROAERO-GENIC	
	Number	Per cent	Number	Per cent	Number	Per cent
Water	32	2.3	61	4.5	13	0.9
Soil............	66	10.7	125	20.2	14	2.3
Grains	85	16.5	212	41.5	0	0.0
Feces	14	1.3	12	1.1	0	0.0

normal strains, however, only two were *Escherichia* with respect to the IMViC reactions. The same holds true of all other sources; abnormal fermenters were of the *Aerobacter* and intermediate groups with the exception of two anaerogenic type 19's and one type 14 from soil. Inasmuch as these were from areas exposed to fecal contamination their possible fecal origin cannot be denied.

Since a number of taxonomists consider it advisable to distinguish between *Aerobacter aerogenes* and *Aerobacter cloacae*, a number of cultures of type 2 (typical *Aerobacter*) and of type 1 were examined for motility and gelatin liquefaction. Table 7 shows that 8.5 per cent of the type 2 cultures are irregular with regard to the concept of positive correlation of gelatin and motility, as are 53.4 per cent of type 1 cultures.

Subsequent to the conclusion of this study the new edition

(1939) of "Bergey's Manual of Determinative Bacteriology" was released. In the new edition the distinction between the two species is retained, but the fundamental separation is based on the ability of *Aerobacter aerogenes* to produce gas from glycerol while *Aerobacter cloacae* does not. Typical strains remain as before with respect to motility and gelatin liquefaction but occurrence of motile strains of *aerogenes* and occasional gelatin-deficient *cloacae* strains is recognized. Ninety-four *Aerobacter* strains (type 2) from various sources were available. These were tested for glycerol fermentation, motility, and gelatin liquefaction.

TABLE 7

Classification of Aerobacter strains with respect to motility and gelatin liquefaction

SOURCE	NUMBER	$\begin{matrix}+\bullet\\+\dagger\end{matrix}$	$\begin{matrix}+\\-\end{matrix}$	$\begin{matrix}-\\+\end{matrix}$	$\begin{matrix}-\\-\end{matrix}$
Type 2:					
Soil........	207	34.5	2.0	6.5	57.0
Feces.....................	39	15.4	2.6	7.7	74.3
Total	246	31.3	2.0	6.5	60.2
Type 1:					
Soil	17	0.0	52.9	0.0	47.1
Feces.............	13	0.0	53.9	0.0	46.1
Total	30	0.0	53.4	0.0	46.6

* Top row of signs, gelatin liquefaction.
† Lower row of signs, motility.

Of the cultures listed in table 8 the most interesting are the anomalies. Motility clearly does not offer a satisfactory criterion for separation of species since 38.9 per cent of the *aerogenes* strains are motile. Gelatin liquefaction is somewhat better, there being only 9.6 per cent of anomalous cultures when it is paired with motility in the determination of *cloacae*. The total percentage of anomalies in this series (28.5) is, however, somewhat greater than that of the preliminary series (8.5). This is probably due to the fact that gelatin observations on the first series were terminated after 15 days while in some cases in the second series the tests were retained for 40 days. Had the first series been

similarly extended it is quite likely that more non-motile gelatin liquefiers would have been found. The use of glycerol fermentation as the primary characteristic seems to be most suitable. However, since gas production is the cardinal distinction the differentiation is subject to the same difficulties as have been reported in lactose fermentation, namely delayed reactions. In a number of instances gas was produced only after 21 days incubation, and only a single culture failed to produce acid. Basing the distinction solely on gas production from glycerol, it is

TABLE 8

Classification of Aerobacter strains with respect to glycerol fermentation, gelatin liquefaction, and motility

SOURCE	NUM-BER	AERO.		CLOACAE		IRREG.		
		+* −† −‡	+ − +	− + +	A + +	+ + +	+ + −	A + +
Milk	1	0.0	0.0	0.0	100.0	0.0	0.0	0.0
Water..	14	21.4	7.2	0.0	42.8	7.2	0.0	21.4
Soils	32	31.2	37.5	0.0	12.5	3.1	15.7	0.0
Grains...	18	11.1	0.0	5.6	83.3	0.0	0.0	0.0
Feces	29	72.5	3.4	0.0	6.9	0.0	13.8	3.4
		38.3	14.9	1.1	29.8	2.1	9.6	4.2
Total	94	53.2		30.9		15.9		

* Top row of signs, glycerol fermentation.
† Middle row of signs, gelatin liquefaction.
‡ Lower row of signs, motility.

possible to classify all cultures as either *aerogenes* or *cloacae*, but even admitting the validity of including motile organisms as *aerogenes* there are still 15.9 per cent of the cultures which do not show the expected correlation among the three reactions.

DISCUSSION

The use of the IMViC differential media for the classification of the members of the coliform group has certain advantages in the study of the relationships of the various members of the group one to the other. In the present study the addition of

cellobiose to the fundamental media has aided immeasurably in formulating the classification chart (fig. 1). As a routine procedure, however, it seems hardly necessary to utilize all of the tests. The indole and Voges-Proskauer reactions are sufficient to classify any strain as to the section of the coliform group to which it belongs. It is true that a similar separation can be made by utilizing the Voges-Proskauer and citrate reactions, and that the general distribution of organisms will not be significantly altered. On the other hand the indole test is less expensive in both time and materials, and the greater stability of cultures with respect to the indole test is a point in its favor.

Attention has been called to the extreme variability of the coliform group in its distribution as found by various authors. It is unfortunate that this condition should exist, since it has caused considerable divergence of opinion as to the significance of the various sections of the group. The lack of homogeneity exhibited by Table 3 is very significant from the statistical point of view, yet in many instances it can be seen that the high value of chi-square for a given section of the coliform group is due to the introduction of a high component by the results obtained by one or two investigators. The results of the present study are by no means guiltless in this respect. It is not possible to reconcile these variations by simple consideration of the results presented in the literature in most instances, although analysis of Burke-Gaffney's (1932) and Koser's (1924, 1926) figures as well as those of the present study indicates where much of the trouble lies. The best that can be said is that, if a series of cultures is sufficiently large and due care has been taken to make the sampling statistically accurate, the distribution given by any one investigator will be representative of his particular locality.

Analysis of the distribution as given in this paper suggests strongly that *Aerobacter* and intermediate strains constitute the normal coliform flora of non-fecal sources and *Escherichia*, the normal flora of feces. It is the authors' opinion that occurrence of strains outside of these habitats, e.g., *Aerobacter* and intermediates in feces, is adventitious. It is admitted that these groups occur in fecal material just as *Escherichia* are found in soil and

water. They are not, however, representative of a homogeneous population as are *Escherichia*. The ascendance of *Aerobacter* and intermediates over *Escherichia* in feces stored at low temperature as reported by Parr (1938a) seems to us to be an expression of the lower optimum temperature for these groups, which again would suggest their essentially non-fecal origin.

Variability in lactose fermentation must of necessity interest the sanitarian as well as the taxonomist. The significance of abnormal fermenters is still a moot question. Parr (1936, 1938a, b) has made an extensive study of slow fermenters from feces, and it can not be gainsaid that they frequently appear at times of crisis in the fecal flora. From that point of view they are admittedly a sign of potential danger. On the other hand we have encountered few aberrant strains from fecal material, and their incidence in non-fecal sources is extremely high. We prefer to consider the anerogenic strains of *Aerobacter* and intermediates as possible links in a putative "evolutionary" chain. It is of interest to note that slow-fermenters are common among the plant pathogens, and furthermore we have observed a number of strains of *Serratia* which produced as much as 20 per cent gas from lactose at 37°C. when they were first isolated. Hence the dividing line between the true enteric strains and others of the *Enterobacteriaceae* is by no means well defined. The inclusion of anaerogenic strains in the coliform group may well be questioned. We have, however, repeatedly observed complete loss of gas production on the part of some of our cultures. One strain of type 2 was maintained in our culture collection for a period of two years during which time it was frequently checked for constancy of reaction. At the end of this period an anaerogenic variant appeared which was serologically and biochemically identical with the parent strain except in gas production from carbohydrates. Numerous other instances of fluctuation or complete loss of gas production which have been noted will be treated in subsequent papers on the serology of the coliform group.

The type of variability expressed in figure 2 is of more interest to the taxonomist. The most significant observation seems to be the infrequency of shifts from one group to another, i.e., the

stability of the indole reaction as opposed to citrate utilization. When such shifts do occur they tend to be in the basic types of the groups. Parr (1938c) has called attention to a citrate mutation similar to that observed in lactose fermentation by *Escherichia coli-mutabile*. Our own experience has suggested that this variation may be quite common so that considerable confusion might arise in classifying strains. This factor is the more important since the citrate-positive variants are serologically identical with the parent strains as will be shown in a subsequent report. The indole test does not seem to be as susceptible to variation, although we have recently encountered a single strain which gives rise to indole-positive variants in a somewhat similar manner. This abnormality is being subjected to further study.

Separation of the genus *Aerobacter* into the species *aerogenes* and *cloacae* has long seemed to us of questionable value. Kligler (1914), Levine and his associates (1917, 1932, 1934, Johnson and Levine 1917) and others have noted many instances of lack of correlation among fermentation of glycerol, gelatin liquefaction, and motility taken either as a group or in pairs. Our own results confirm their observations, since, even when the existence of motile strains of *aerogenes* is conceded, the percentage of discrepancies is still so high as to make the separation appear artificial. Furthermore a subsequent report will show that there is no serological evidence to support division of the group into species.

A recent report by Perry (1939) suggests that the use of glycerol may have taxonomic significance in another way. Of 45 strains which he classified as *Citrobacter* 42 were indole-negative and fermented glycerol. Three indole-positive strains, which would correspond to our types 14, 15, and 16, failed to ferment glycerol as did 1374 strains classified as *Escherichia*. A correlation study along these lines might shed further light on the relationship of our type 8 to type 2.

SUMMARY

1. The distribution of 6577 strains of coliform organisms in milk, water, soil, grains, and feces suggests that *Aerobacter* and intermediates constitute the normal coliform flora of non-fecal

materials, while *Escherichia* are normal to feces. It is suggested that occurrence of these groups outside these normal habitats is adventitious.

2. Stability of the indole test as compared with citrate utilization renders it more suitable for taxonomic purposes.

3. Most aberrant lactose fermenters appear to be of questionable sanitary significance.

4. There is some question as to the advisability of continuing the distinction between *Aerobacter aerogenes* and *Aerobacter cloacae*.

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ANTIGENIC RELATIONSHIPS OF THE COLIFORM BACTERIA

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The problem of the serological homogeneity of the coliform group is not new. Escherich in 1885 first described a coliform organism from the intestines. Following closely the discovery of the agglutination phenomenon by Gruber and Durham in 1896, Pfaundler (1898) used this reaction to study the serological relationships of the coliform bacteria. He found no serological homogeneity among the coliform cultures; furthermore, he noted that the serums of immunized animals did not always agglutinate their homologous strains. Radzievsky (1899) tested 64 coliform strains isolated from the intestines of the same individual in 8 coliform immune serums. From his results he concluded that coliform bacteria could be divided into a large number of serological groups.

Jatta (1900) produced 16 coliform immune serums in which he tested a number of coliform cultures. He found all strains agglutinated in their homologous immune serums and some cross agglutination to low titer occurred among the heterologous strains. From this he inferred that there were a large number of species, culturally and morphologically identical.

In 1902 Di Donna showed that animals could be immunized to "pathogenic" as well as to "nonpathogenic" coliform bacteria. However, only the homologous strains agglutinated to a high titer. Altman and Rauth (1910) noted that neither age on agar nor colonial variations altered the receptors of coliform organisms. In 1913 Mackie confirmed the preceding results and again emphasized the fact that agglutination of coliform bacteria, with few

exceptions, was limited to the particular strain used for immunization. Six years later, 1919, Van Loghem again confirmed the serological diversity among coliform organisms since he rarely found one coliform culture which would agglutinate in the immune serum of another.

Redman (1922) attempted to classify serologically lactose-fermenting bacteria isolated from cheese, milk and water. He found that some cross agglutination occurred among heterologous strains but nothing that was uniform or that could be correlated with the source from which the cultures were isolated. During the same year (1922) Dudgeon, Wordley and Bawtree, working with hemolytic coliform strains, reported some definite serological grouping. With two exceptions, all their hemolytic strains of coliform organisms were agglutinated by an antiserum prepared from one hemolytic strain. This was not true of non-hemolytic strains. In 1924 Czickeli tried to differentiate serologically between intestinal coliforms and those isolated from the bladder of patients having cystitis, but found no definite serological division.

Meyer and Lowenberg (1924) reported some antigenic relationship among hemolytic coliforms. A large percentage of hemolytic strains agglutinated in immune serums prepared from four hemolytic strains. In nonhemolytic antiserums, however, only the homologous and a few of the hemolytic strains agglutinated. Pesch and Simchowitz (1926) attribute the variability of coliform agglutination reactions to the electrical charge of the strain concerned. Among 22 strains used in this investigation the hemolytic strains were inclined to agglutinate more strongly than the nonhemolytic strains, due, according to the authors, to a reduced electric charge. Hees (1926) found that he could not predict whether a strain would agglutinate, even in the antiserum of a culture from the same source. Strunz (1926) immunized rabbits with 14 of 23 strains of fecal coliform cultures and by cross agglutination classified these 23 strains into 3 major groups independent of their origin.

Meyer (1929) divided *Escherichia coli* into 3 types on the basis of type-specific and species-specific antigens. Both antigens

occurred in hemolytic strains with only the species-specific antigens occurring in the nonhemolytic strains. Hemolytic strains could be grouped according to their type-specific antibodies into two definite groups. Nonhemolytic strains reacted with hemolytic antiserums, due to the species-specific antibody held in common by them both. Meyer regards *Escherichia coli* as being analogous to pneumococcus although serologically the former exhibits the more diverse reactions. Harada (1929) attempted to confirm Meyer's hypothesis. He found that serums from patients with hemolytic coliform urinary infections agglutinated strains of hemolytic types more strongly than others. This indicated to Harada, as to Meyer, the presence of both type and species-specific antigens in hemolytic strains.

Edwards (1929) was among the first to work on *Aerobacter aerogenes*. His cultures were isolated from milk, feces, soil and water. He was unable to find any biochemical or serological test which could be used to differentiate these organisms. Five of the *Aerobacter aerogenes* cultures were serologically identical with "*B. Friedlander*" and two were identical with a strain of the "granuloma bacillus."

Gundel (1930) failed to confirm Meyer's and Harada's findings on the serological relationships of hemolytic and nonhemolytic coliform strains. By means of agglutination and adsorption tests with hemolytic and nonhemolytic strains he found no uniform serological relationship as reported by Meyer (1929) or Harada (1929).

Bruschettini (1936) attempted to differentiate serologically between coliform strains of human and animal origin. With 40 immune serums prepared from hemolytic and nonhemolytic coliform strains of herbivorous and carnivorous animals, including man, he was unable to demonstrate any serological grouping with respect to source. He also opposed Meyer's and Harada's theories on the serological behavior of hemolytic and nonhemolytic coliform strains.

In 1937 Magheru and coworkers studied the somatic antigen of coliform organisms. They found that the entire "O" antigen of *Escherichia coli* was composed of a sugar-lipoid complex and that

not all variants of the same strain contained the complete antigen. Precipitin reactions between complete antigens and anti-bacterial serums indicated that *Escherichia coli* consists of many serological varieties.

Julianelle (1937), working with *Aerobacter aerogenes,* found in 3 "substrains" three type-specific immunological entities. One strain reacted only with its own antiserum, a second strain was related to pneumococcus II and the third strain took part in reciprocal reactions with both pneumococcus II and *"Friedlander's bacillus."* Julianelle believed that these relationships depended upon the chemical constitution of the capsular carbohydrates.

The controversy regarding the classification of that group of bacteria intermediate between *Aerobacter* and *Escherichia* is too well known to require reviewing. In the fifth edition of Bergey's Manual (1939) the intermediates are recognized as a species *freundii* (IMVIC $- + - + +$, type 8) in the genus *Escherichia* together with *Escherichia coli* (IMVIC $+ + - - -$, type 19). It is true that intermediates are found in feces but in our work on approximately 600 fecal specimens they seem to occur even less frequently than *Aerobacter.* In a large majority of bacteriology textbooks the natural habitat of the intermediates as of *Aerobacter* is stated to be cereals and soils. On the basis of the Eijkman test, the intermediates appear to be more closely related to *Aerobacter* than to *Escherichia,* since gas production by the intermediates is, for the most part, inhibited at 46°C. (Perry, 1939).

This brief but representative survey of the literature shows little or no serological homogeneity in the coliform bacteria. However, most authors concluded that further work in this field was necessary before any definite conclusions could be formulated. Early investigators naturally did not differentiate between *Aerobacter,* intermediates and *Escherichia.* Some of the more recent serological investigations have been made on either *Aerobacter* or *Escherichia* organisms but, to our knowledge, no general work has been attempted on the serological relationships of the three sections of the coliform bacteria. A part of the present work dealing with the antigenic relationships between the

Aerobacter, intermediate and *Escherichia* groups will be discussed under the heading, *Antigenic Relationships of the Coliform Group*.

Any serological investigation of the coliform group should also consider the "paracolon" organisms.   Topley and Wilson (1937) have reserved the term "paracolon" for coliform-like organisms (IMVIC + + − − −) frequently isolated from man during gastro-intestinal disorders, which either do not ferment lactose or ferment it slowly with the formation of acid or acid and gas.   The serological relationships of a number of paracolon cultures isolated from man during food infection epidemics have been studied and will be discussed under the heading, *Antigenic Relationships of the Paracolon Group*.

In the course of our work on over 10,000 coliform cultures, variants, differing biochemically from the parent culture, have occasionally been isolated.   The antigenic relationship of variant to parent was in some cases determined and will be discussed under *Antigenic Relationships between Biochemically Different Variants of Single Cultures*.

ANTIGENIC RELATIONSHIPS OF THE COLIFORM GROUP

As previously reported (Stuart, Griffin and Baker, 1938) and (Griffin and Stuart, 1940), more than 6000 coliform cultures isolated from soil, water, cereals, milk and feces were classified according to their reactions in indole, methyl red, Voges-Proskauer, citrate and cellobiose mediums.   These cultures fell into 3 groups (A, B and C) with 6 types in each group and one type intermediate between two of the groups.   In each group there was what has been termed a basic type.   For example, of 2617 group A cultures 2512 were type 2 and 105 were types 3 to 7.   Type 8 was the basic type in group B and type 19 in group C.   There was also a tendency for the type farthest removed biochemically from the basic type, that is 7, 13 and 14, to be the second most important type in a group from the numerical viewpoint.

The problem of antigenic relationships in the coliform bacteria was first approached by preparing in rabbits monovalent anti-serums against living cultures of types 1, 2, 7, 8, 13, 14 and 19.

From 3 to 10 heterologous cultures of each of the 19 different types were tested for agglutination in each immune serum. From the results of these tests the heterologous antigenicity of the coliform bacteria was immediately apparent. Of 10 type 2 cultures tested in the type 2 antiserum, only two agglutinated. Some of the type 2 cultures which were negative in the type 2 antiserum did agglutinate to low titers in the antiserums of types from group B or C. With the exception of type 1 and type 5 cultures, which showed some evidence of antigenic homogeneity, all other types reacted in the different serums much like type 2. Of 33 type 1 cultures, 49 per cent agglutinated to an average titer of 690 in a type 1 serum with an homologous titer of 1280. Nine type 5 cultures isolated from water, milk and feces all agglutinated in a single type 5 antiserum but with marked variation in titer.

Despite the antigenic heterogeneity of the coliform group demonstrated by this work, there was a tendency on the part of the cultures from any group to agglutinate more frequently and to somewhat higher titers in the antiserums of that group than in the antiserums of the other two groups. It was therefore decided to limit the investigation to types 2, 8 and 19 so that a large number of cultures of the three types could be tested in several immune serums of each type.

Methods

Monovalent immune serums were prepared in rabbits with living cultures of nine type 2, nine type 8 and ten type 19 cultures. To determine whether there was any correlation between source and serological relationships, cultures used for immunization were selected so that with each type (2, 8 and 19) at least two animals were immunized with strains isolated from water, soil, milk and feces.

The serums of animals to be immunized were tested for normal agglutinins against a number of coliform cultures from the different groups. Agglutinins were present in some of the animals with titers ranging from 1:2 to 1:40. To avoid confusion resulting from the presence of normal agglutinins, cross-agglutination tests with immune serums were started in a dilution of 1:50.

(Hereinafter all dilutions and titers will be referred to by their reciprocals.) Because of the large number of tests to be conducted, serum dilutions of 50, 250 and 1250 were used. From these dilutions, titers were computed as follows: if the degree of agglutination in the 50 dilution was $++$ or $+$ and there was no reaction in the 250 dilution, the titer of the culture was recorded as 50; if the degree of agglutination in the 50 dilution was $++++$ or $+++$ and there was no agglutination in the 250 dilution the titer was recorded as 150, the mean of the two dilutions involved, etc. Any culture agglutinating in the 1250 dilution of any serum was tested in regular serial dilution so that its titer could be accurately compared with the titer of the organism used to produce the immune serum.

To check this method of "computing" titers, 50 cultures agglutinating in one or more of the antiserums were tested in the usual serial dilutions starting with 50. The average titer of these cultures obtained from the "actual" titer was 1400 and from the "computed" titer 1260. The difference between the two average titers, 140, represents a maximum variation of but one-fifth of one dilution above or below the average titer and cannot be significant, since the same organisms tested in the same antiserum on different occasions may vary by as much as one whole dilution.

Twenty-two pseudomicro-aerogenic type 2 cultures, cultures producing acid and gas more readily at 20°C. than at 37°C., (Stuart, Mickle and Borman, in press) were tested for cross agglutination in the nine type 2 antiserums. Twenty-eight *Erwinia* cultures were tested in the antiserums of the three types. From 50 to 113 type 2, 8 and 19 cultures were tested in all type 2, 8 and 19 antiserums and 22 type 13 cultures were tested in all type 8 and 19 antiserums. The cultures used in the cross-agglutination tests were carefully selected so that all sources were represented and so that no two cultures of the same type came from the same sample or, for the most part, from the same supply of any source. Selection on this basis was made when preliminary experiments showed that many of the cultures of the same type from one sample were antigenically identical.

The percentage of cultures agglutinating in each antiserum

and the average titer obtained from the computed titers of each antiserum are graphically summarized in figure 1 and, with some exceptions, tabulated in tables 1, 2, 3 and 7.   In these tables and figure 1 the percentage of a specified type of culture agglutinating in all the antiserums of any one type does not represent

FIGURE 1

Summary of the reactions of the different types of cultures in the different types of antiserums

NUMBER OF CULTURES TESTED	PER CENT AGGLUTINATING	TITERS IN TERMS OF 100
		1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
		In nine type 2 antiserums, average homologous titer 9,244
28 Erwinia	16.3	
22 pseudo.*	19.2	
103 type 2	16.9	
50 type 8	11.7	
50 type 19	9.5	
		In nine type 8 antiserums, average homologous titer 11,793
28 Erwinia	10.7	
50 type 2	13.7	
108 type 8	7.4	
22 type 13	15.8	
50 type 19	10.6	
		In ten type 19 antiserums, average homologous titer 28,160
28 Erwinia	6.3	
50 type 2	15.2	
50 type 8	19.6	
22 type 13	26.0	
113 type 19	38.4	

Pseudo.* = pseudomicro-aerogenic.

the actual percentage of cultures agglutinating but rather the incidence of agglutination; for example, 28 *Erwinia* cultures were tested in nine type 2 antiserums with a total of 252 tests of which 16.3 per cent of the 252 tests were positive.

Antigenic relationships between cultures agglutinating in the different antiserums were determined by adsorption.   Thick

agar plates were streaked with swabs heavily impregnated with the growth from 24-hour agar slants. After 24 hours incubation at 37°C. the growth was removed with 2 ml. of saline per plate. The organisms were packed by high speed centrifugation, the supernatant fluid discarded and the serum dilution added to the packed organisms. The growth from one plate was used for each 2.5 ml. of serum dilution. The first adsorption was done at 37°C. for one hour, the second for one and one-half hours and the third for two hours. After the third adsorption the antiserum was tested with the specified cultures. In reciprocal adsorptions between two or more cultures, the same technic was used, except that the antiserum was tested after each adsorption.

For example, a type 2 agglutinated 17 type 2 cultures with titers ranging from 50 to 1250. The antiserum in a 1:10 dilution was adsorbed with one of the cultures agglutinating to 1250. The adsorbed serum was tested in regular serial dilutions (starting with 1:50) with all 17 organisms which had originally agglutinated in this serum, including the homologous culture. Agglutinins remained in the serum for the homologous and 5 of the heterologous cultures. The serum was then adsorbed with the highest titering of the five heterologous cultures and tested with all cultures which had agglutinated in the serum after adsorption with the first culture. This procedure was continued until all the heterologous agglutinins were removed. This was done with all type 2 and 8 cultures agglutinating in their respective antiserums. Type 19 cultures agglutinating in type 19 antiserums were treated quite differently as will be described later. Any antiserum agglutinating a type heterologous culture to 1250 or higher was adsorbed with the culture to determine the antigenic relationship of the agglutinating culture to the homologous.

The motility of all type 2, 8 and 19 cultures used in this work was determined. Five type 2, five type 8 and six type 19 antiserums were prepared from motile cultures.

None of the 103 type 2 cultures gave hemolysis on rabbit-blood agar plates while 47 per cent of the 113 type 19 cultures were weakly or moderately hemolytic. Type 19 cultures isolated

from feces were no more hemolytic than cultures isolated from water, milk or soil. Four of the type 19 antiserums were produced with hemolytic cultures.

All type 2 and 19 cultures were grouped according to their reactions in sucrose, salicin, dulcitol and adonitol when first isolated. At varying intervals during the two years in which this work was in progress the type 19 cultures were plated, a single colony fished to an agar slant and its fermentative reactions retested. The cultures were plated and tested eight times and only 61 of the 113 cultures were perfectly consistent in their reactions over the two years. In many cases the changes observed were slight, such as in one culture which produced acid and at least 20 per cent gas in sucrose on seven tests and only a bubble of gas on the other test. In other cases the change was marked, as in one culture which had produced acid and gas in sucrose, salicin and dulcitol, but on subsequent tests fermented only salicin. Most cultures showed changes in reaction on only one or two tests. Two cultures, however, never gave the same reactions on any two consecutive tests. Changes in reaction occurred most frequently in dulcitol and sucrose. Of 57 cultures fermenting dulcitol 32 showed variation in this medium; of 38 cultures reacting in sucrose, 16 varied while only 9 of the 58 cultures fermenting salicin varied.

Results

The work in the three sections of this paper involved the immunization of 139 rabbits, over 8000 agglutination, several hundred adsorption, and numerous motility, carbohydrate and hemolytic tests. Moreover, some of the tests were repeated on several occasions over a period of two years. Many interesting facts apart from serological relationships were observed but it is deemed advisable to present only those facts which tend toward a better understanding of the antigenic relationships within the coliform group and of the coliform group to closely related groups of bacteria. To conserve space, tables on adsorption tests and tables on the correlation of motility, carbohydrate reaction and source with agglutination have been eliminated.

Pseudomicro-aerogenic type 2 cultures: Of 22 cultures tested in the nine type 2 antiserums 19.2 per cent agglutinated to an average titer of 534 (fig. 1). Only two of these cultures agglutinated to a titer of 1250 and they failed to reduce the homologous titer of the antiserums upon adsorption. The percentage (19.2) of pseudomicro-aerogenic cultures agglutinating in type 2 antiserums is approximately equal to the percentage (16.9) of type 2 cultures agglutinating in the same antiserums. Moreover, since the average titer (534) of the pseudomicro-aerogenic cultures is approximately one half the average titer (1148) of the type 2 cultures, it would seem that antigenically the two kinds of cultures were quite closely related.

Erwinia cultures. The similarity of the biochemical reactions of organisms of the *Erwinia* genus and the coliform organisms has been pointed out by Rahn (1937) and others. In the fifth edition of Bergey's Manual, *Serrateae*[1] and *Erwineae* appear as tribes under the family *Enterobacteriaceae*. This grouping of *Erwineae* can be justified from a serological viewpoint since 16.3 per cent of 28 known plant pathogens (supplied by Perry Elrod of Ohio State University) agglutinated in the nine type 2 antiserums to an average titer of 475 (fig. 1). The percentage of

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[1] We have isolated from milk 8 cultures of *Serratia* producing 20 per cent or more gas in lactose broth in 24 to 48 hours on the first transplant to the sugar medium. Five of these cultures were nonpigmented when first isolated but recovered their ability to produce pigment after successive transplants on agar slants incubated at 20°C. Eight other *Serratia* cultures, six isolated from widely different sources of water and 2 from different human fecal specimens, are being studied. Four strains from water produced acid and 20 per cent gas in lactose after serial transplants in this medium for from 2 to 3 months. Two strains produced only slight acid after 6 months of serial transplants in lactose broth. One fecal strain produced acid and gas in lactose on the first transplant and the other, only slight acid after 6 months of successive transplants. All 16 *Serratia* cultures have the IMVIC reactions of type 2 but cultures producing acid and gas in lactose are strongly positive in the Voges-Proskauer reaction and produce a heavy growth on citrate in 24 hours while cultures producing only acid are weakly V-P positive and grow sparsely on citrate in 24 hours. Considering the small number of cultures, this may be mere coincidence or it may represent a series of metabolic intergrades within the group. It is hoped that a sufficient number of *Serratia* cultures can be collected to test their antigenic relation to the coliform group. In any event, we agree with the American Committee on Classification that organisms in the genus *Serratia* are closely related to the coliform bacteria.

Erwinia cultures agglutinating in type 2 antiserums is almost identical with the percentage of type 2 cultures agglutinating in the same antiserums but the average titer of the *Erwinia* cultures is approximately half that of the type 2 cultures. The *Erwinia* cultures were also tested in nine type 8 antiserums and ten type 19 antiserums. Of the 28 cultures 10.7 per cent agglutinated in the type 8 antiserums to an average titer of 297 and 6.3 per cent in the type 19 antiserums to an average titer of 190. The steadily decreasing percentage of cultures agglutinating, and the decreasing average titer of these cultures in type 2, 8 and 19 antiserums, may merely represent type distribution of *Erwinia* cultures since, according to IMVIC reactions, seventeen of the 28 cultures were type 2, ten type 8 and one type 19. Three cultures agglutinating to 1250 or higher in type 2 antiserums failed to reduce the homologous titers of the antiserums upon adsorption.

It can be said that certain *Erwinia* cultures are closely related to certain coliform organisms but whether the *Erwinia* group is more closely related to type 2 than to type 8 and 19 coliform cultures cannot be determined without further work.

Type 2 cultures. Of 103 such cultures exclusive of cultures used to prepare the antiserums, 16.9 per cent agglutinated in nine type 2 antiserums to an average titer of 1148 (table 1). When the percentage of cultures agglutinating in two or more antiserums was approximately equal it was not due, for the most part, to the same cultures agglutinating in these antiserums. Only one of the cultures agglutinated to the homologous titer in one of the antiserums. Some cultures agglutinated in a single antiserum, others in from two to six antiserums and some failed to agglutinate in any antiserum.

Fifty of the 103 type 2 cultures were tested in all type 8 and 19 antiserums. In the type 8 antiserums, 13.7 per cent of the cultures agglutinated to an average titer of 397 while, in the type 19 antiserums, 15.2 per cent agglutinated to an average titer of 232. The high percentage of cultures agglutinating in the type 19 antiserums was somewhat surprising, but figure 1 shows that except for *Erwinia*, type 19 antiserums agglutinated a higher percentage of heterologous type cultures than any other type of antiserums.

The average titer of type 2 cultures in type 8 antiserums (397) and in type 19 antiserums (232) might indicate that type 2 is antigenically more closely related to type 8 than to type 19.

Adsorption tests were made with the type 2 cultures as previously described. All heterologous agglutinins were completely

TABLE 1

Agglutination of type 2 cultures in type 2, 8 and 19 antiserums

100 TYPE 2 CULTURES	IN NINE TYPE 2 ANTISERUMS, AVERAGE HOMOLOGOUS TITER 9,244									
	1	2	3	4	5	6	7	8	9	10
Per cent agglutinating	16.5	24.5	20.3	13.5	22.3	21.3	27.1	6.7	9.7	
Average titer	650	236	236	292	2471	1872	1557	164	1305	
Per cent agglutinating in all antiserums.. .. .. 16.9										
Average titer in all antiserums 1148										

50 TYPE 2 CULTURES	IN NINE TYPE 8 ANTISERUMS, AVERAGE HOMOLOGOUS TITER 11,793									
Per cent agglutinating	12.0	12.0	18.0	6.0	16.0	16.0	8.0	18.0	18.0	
Average titer .	766	433	277	516	262	362	112	305	627	
Per cent agglutinating in all antiserums 13.7										
Average titer in all antiserums 397										

50 TYPE 2 CULTURES	IN TEN TYPE 19 ANTISERUMS, AVERAGE HOMOLOGOUS TITER 28,160									
Per cent agglutinating	30.0	10.0	14.0	26.0	16.0	16 0	14.0	10.0	10.0	6.0
Average titer	366	170	307	273	175	150	121	170	150	183
Per cent agglutinating in all antiserums . . 15.2										
Average titer in all antiserums 232										

removed from some type 2 antiserums after three adsorptions with one heterologous culture. Other antiserums had to be adsorbed with two and some with three cultures to remove all the heterologous agglutinins. No antiserum was deprived of its homologous agglutinins; in fact, the greatest reduction in the homologous titer was from 10,240 to 2560 in a serum which had been adsorbed

nine times with three different cultures and stored for one day in the ice box between each series of adsorptions. This indicates that of 918 chances (103 cultures times 9 antiserums minus 9 homologous cultures) not one culture encountered was sufficiently related to any of the 9 organisms used for immunizing to remove the homologous agglutinins. At this point, it should be recalled that the 103 cultures were selected so that no two cultures came from the same sample or, for the most part, from different samples of the same source. Several type 2 cultures agglutinated to titers of 1250 or higher in type 8 and 19 antiserums but failed to reduce the homologous titer of the antiserum upon adsorption.

The work on type 2 cultures had been completed when the fifth edition of Bergey's Manual was released. All 9 cultures homologous to the type 2 antiserums and 59 of the heterologous type 2 cultures were available. These cultures were tested in glycerol and gelatin and the cultures classed as *A. aerogenes* or *A. cloacae*. Five antiserums had been prepared from cultures with the reactions of *A. cloacae*, three from *A. aerogenes* and one from a culture producing acid and gas from glycerol and liquefying gelatin. There was, however, no correlation between agglutination and the grouping of the type 2 cultures according to their reactions in glycerol and gelatin.

Type 8 cultures. Of 108 cultures, exclusive of those used to prepare the antiserums, 7.4 per cent agglutinated to an average titer of 1572 (table 2) in the nine type 8 antiserums. Fifty of the type 8 cultures were tested in all type 2 and 19 antiserums. In the type 2 antiserums, 11.7 per cent agglutinated to an average titer of 393 while 19.6 per cent agglutinated to an average titer of 412 in type 19 antiserums. Here again a higher percentage of cultures agglutinated in the type 19 antiserums than in the type 2 and 8 antiserums. On the basis of average titer, however, type 8 appears to be antigenically intermediate between types 2 and 19.

As previously pointed out, any culture agglutinating to 1250 or more in any antiserum was retested in regular serial dilutions. Twenty-six type 8 cultures were so tested in type 8 antiserums. Eleven cultures agglutinated to the homologous titer or to within one or two dilutions of the homologous titer and were used to

adsorb the antiserum in which they agglutinated.   Four strains agglutinated to the homologous titer in one antiserum.   The four strains were antigenically identical or closely related to each other and to the strain used to produce the antiserum since any one of the four strains completely removed all agglutinins including the

TABLE 2

Agglutination of type 8 cultures in type 2, 8 and 19 antiserums

50 TYPE 8 CULTURES		IN NINE TYPE 2 ANTISERUMS, AVERAGE HOMOLOGOUS TITER 9,244									
		1	2	3	4	5	6	7	8	9	10
Per cent agglutinating		24.0	8.0	8.0	18.0	14.0	8.0	12.0	10.0	4.0	
Average titer		491	1325	75	383	2214	175	250	610	100	
Per cent agglutinating in all antiserums	11.7										
Average titer in all antiserums	393										
108 TYPE 8 CULTURES		IN NINE TYPE 8 ANTISERUMS, AVERAGE HOMOLOGOUS TITER 11,793									
Per cent agglutinating		4 6	7.4	4.6	2.7	10.1	10.1	4.6	5.5	16.6	
Average titer		510	3625	430	850	6980	668	2050	316	383	
Per cent agglutinating in all antiserums	7.4										
Average titer in all antiserums	1572										
50 TYPE 8 CULTURES		IN TEN TYPE 19 ANTISERUMS, AVERAGE HOMOLOGOUS TITER 28,160									
Per cent agglutinating		18.0	24.0	34.0	22 0	16.0	18.0	14 0	18.0	24 0	8.0
Average titer		314	667	6227	293	303	464	133	364	379	361
Per cent agglutinating in all antiserums	19.6										
Average titer in all antiserums	412										

homologous agglutinins from the antiserum.   Another type 8 culture M524 agglutinated to the homologous titer of an antiserum prepared from culture M10.   An antiserum was prepared with M524 and reciprocal adsorptions showed the two cultures to be antigenically identical.   Both strains came from the same milk supply but were isolated two months apart.

The six other type 8 cultures agglutinated to within two or three dilutions of the homologous titer of one or another of the type 8 antiserums. In four cases the homologous titers were significantly reduced while in the other two cases the homologous titers were not altered by adsorption. General adsorptions, that is, removal of all heterologous agglutinins by adsorption with one, two or more of the cultures agglutinating in a serum, failed to reveal any more cultures capable of reducing the homologous titer. No type 8 culture agglutinating to 1250 or more in type 2 or 19 antiserums was able to alter the homologous titer of the serum upon adsorption. It would seem then that the type 8 cultures were less diversified than the type 2 cultures since no type 2 culture was found capable of reducing the homologous titer of the type 2 antiserums significantly.

Type 19 cultures. Of 113 such cultures, exclusive of those used to produce the antiserums, 38.4 per cent agglutinated to an average titer of 1261 in the type 19 antiserums (table 3). This is the highest percentage of any type of cultures agglutinating in any type of antiserums. Of 50 of these type 19 cultures, 9.5 per cent agglutinated to an average titer of 307 in type 2 antiserums and 10.6 per cent agglutinated to an average titer of 373 in type 8 antiserums.

Type 19 antiserums agglutinated 92 type 19 cultures sufficiently highly to warrant adsorption of the antiserums. In seven instances the homologous agglutinins were completely removed from the antiserums by the adsorbing strain. In 38 instances the homologous titers were significantly reduced, while in 47 instances the homologous titers were not altered by adsorption with the heterologous cultures. It should be noted that of 918 chances of obtaining type 2 cultures capable of removing or reducing the titers of type 2 antiserums, none was realized; of 963 chances with type 8 cultures and antiserums 1.24 per cent of the opportunities was realized whereas, with type 19 cultures and antiserums 4.01 per cent was realized.

No type 19 culture agglutinating to 1250 or higher in the type 2 and 8 antiserums altered the homologous titer of the antiserum upon adsorption. Because of the relatively high percentage

(38.4) of type 19 cultures agglutinating in their respective antiserums, (one antiserum agglutinating 54 cultures) general adsorptions as previously described were not done.   It seemed desirable, however, to obtain some information regarding antigenic fractions and their distribution in type 19 cultures.

TABLE 3

Agglutination of type 19 cultures in type 2, 8 and 19 antiserums

50 TYPE 19 CULTURES		IN NINE TYPE 2 ANTISERUMS, AVERAGE HOMOLOGOUS TITER 9,244									
		1	2	3	4	5	6	7	8	9	10
Per cent agglutinating ..		20.8	4.7	2.0	4.7	14.6	6.3	8.3	25.0	4.7	
Average titer . .		860	50	250	50	378	183	50	166	450	
Per cent agglutinating in all antiserums . .	9.5										
Average titer in all antiserums .	307										
50 TYPE 19 CULTURES		IN NINE TYPE 8 ANTISERUMS, AVERAGE HOMOLOGOUS TITER 11,793									
Per cent agglutinating		4 0	18 0	2.0	.00	10.0	14.0	8.0	12.0	14.0	
Average titer ..		50	338	50		109	570	550	383	570	
Per cent agglutinating in all antiserums .	10.6										
Average titer in all antiserums .	373										
113 TYPE 19 CULTURES		IN TER TYPE 19 ANTISERUMS, AVERAGE HOMOLOGOUS TITER 28,160									
Per cent agglutinating .		39.4	33.3	40.3	48 2	46.4	37 7	32.4	30.7	40 3	35.0
Average titer . . .		1195	1257	1752	590	625	984	805	2392	958	856
Per cent agglutinating in all antiserums .	38.4										
Average titer in all antiserums . .	1261										

An antigenic analysis of the 10 cultures used to produce the 10 immune serums was not feasible since only 3 of the cultures agglutinated in any of the heterologous antiserums.   Accordingly, 10 of the one hundred and thirteen cultures agglutinating in the greatest number of the ten antiserums were selected for the antigenic analysis.   Each culture was tested in each of the

serums in regular serial dilutions from 20 to the homologous titer
of the serum.   Attempts to determine the number of antigenic
fractions on the basis of actual titers did not seem feasible.   When
a culture agglutinated in one serum to 80 and in another serum
to 5120, a definite quantitative or qualitative antigenic difference
was obvious, particularly when both antiserums had the same
homologous titer.   When, on the other hand, the titers of one
culture in three antiserums were 160, 320 and 640, it was difficult
to draw the line between experimental error and antigenic differ-
ences.   Therefore, the reactions of the 10 cultures in the 10 anti-

TABLE 4

Agglutination of the ten selected type 19 cultures in ten heterologous type
19 antiserums

CUL-TURES	ANTISERUMS									
	A	B	C	D	E	F	G	H	I	J
1	+	0	+	+	+	+	+	+	0	+
2	+	+	+	+	+	+	+	0	0	+
3	+	0	+	+	+	+	+	0	+	+
4	0	+	0	+	+	+	+	0	+	+
5	+	+	+	+	+	+	+	+	+	+
6	+	+	+	+	+	+	+	+	0	+
7	+	+	+	+	0	+	+	+	0	+
8	+	+	+	+	+	+	+	+	+	+
9	+	+	0	+	+	0	0	+	+	+
10	+	0	0	+	0	+	+	+	+	0

+, agglutination; 0, no agglutination.

serums are simply recorded as positive or negative in table 4.
In this table, for convenience, the cultures are recorded as 1–10
and the antiserums as A–J.   None of the cultures selected for
this experiment was used to produce any of the antiserums; fur-
thermore, no culture selected agglutinated to the homologous
titer in any antiserum.

Table 4 shows that culture 1 agglutinated in serum A but not
in serum B which demonstrates that culture 1 had an antigenic
component in common with the culture used to produce anti-
serum A.   Culture 2 agglutinated in both antiserums A and B.
That antigenic component of culture 2 agglutinating in anti-

serum B is not present in culture 1 whereas that component of culture 2 agglutinating in antiserum A may or may not be the same as that of culture 1 agglutinating in antiserum A. Cultures 1 and 2 then possess a minimum of 2, or a maximum of 3, qualitatively or quantitatively different antigenic components with respect to antiserums A and B. Analyzed on the same basis, the ten cultures in the ten antiserums show a minimum of 9 and a maximum of 79 antigenic fractions. Culture 8 agglutinated to within two dilutions of the homologous titer in antiserum A. The homologous titer of this serum was markedly reduced but not completely removed when adsorbed with culture 8. Since no other culture agglutinated to within four dilutions of the homologous titer of any serum, it appears safe to assume that the minimum of nine antigenic fractions shown in table 4 is in addition to the antigens peculiar to the strains used for immunizing.

To study further the antigenic fractions of these cultures, all ten antiserums were adsorbed with culture 1 and each serum tested with all cultures which had agglutinated in the antiserums before adsorption. It was hoped that adsorbing the serums with one culture would reduce the number of cultures agglutinating in each serum to the point where the different antigenic fractions could be determined easily by continued adsorption with one, two or even three additional cultures. Table 5, however, shows that culture 1 was incapable of removing the agglutinins for more than three of the nine remaining cultures from any antiserum. In view of these findings the original plan for determining the actual number of antigenic components in the 10 cultures had to be abandoned because the large number of adsorptions indicated would soon have exhausted the supply of antiserums. However, serum D which originally agglutinated all ten cultures and lost agglutinins for only two cultures after adsorption with culture 1 was adsorbed with the other eight cultures. Tables 5 and 6 show that culture 1 removed from antiserum D agglutinins for cultures 1 and 2 while culture 5 removed from the same antiserum agglutinins for cultures 4, 5 and 6, and culture 6 removed agglutinins for cultures 5 and 6. Agglutinins for all other cultures were removed only by their respective cultures. Serum D, then, which

agglutinated all ten cultures, would have to be adsorbed with seven of the ten cultures to remove completely all the heterolo-

TABLE 5

Agglutination of the ten selected type 19 cultures after the adsorption of the ten heterologous antiserums with culture 1

CUL-TURES	ABSORBED ANTISERUM									
	A	B	C	D	E	F	G	H	I	J
1	−	0	−	−	−	−	−	−	0	−
2	−	+	−	−	+	+	−	0	0	−
3	+	0	−	+	+	+	+	0	+	−
4	0	+	0	+	−	+	+	0	+	+
5	+	+	+	+	+	+	+	+	+	+
6	+	+	+	+	+	+	+	+	0	+
7	+	+	+	+	0	+	+	+	0	+
8	−	+	+	+	+	+	+	−	+	+
9	+	−	0	+	+	0	0	−	+	−
10	+	0	0	+	0	−	−	−	−	−
H	+	+	+	+	+	+	+	+	+	+

+, agglutinins not completely adsorbed; −, agglutinins completely adsorbed; 0, agglutinins not present before adsorption; H, cultures homologous to the antiserums.

TABLE 6

Adsorption of antiserum D with all the selected cultures agglutinating in this antiserum after adsorption with culture 1

TESTED WITH CULTURES	ABSORBED WITH CULTURES							
	3	4	5	6	7	8	9	10
3	−	+	+	+	+	+	+	+
4	+	−	−	+	+	+	+	+
5	+	+	−	−	+	+	+	+
6	+	+	−	−	+	+	+	+
7	+	+	+	+	−	+	+	+
8	+	+	+	+	+	−	+	+
9	+	+	+	+	+	+	−	+
10	+	+	+	+	+	+	+	−
H	+	+	+	+	+	+	+	+

+, agglutinins not completely adsorbed; −, agglutinins completely adsorbed; H, cultures homologous to the antiserums.

gous agglutinins.   A type 2 antiserum agglutinating twenty-seven type 2 cultures was deprived of all heterologous agglutinins after adsorption with but three of the 27 cultures.

The ten antiserums were also adsorbed with culture 2 and tested as in the previous experiment and serum G treated in the same manner as serum D.   On the basis of these adsorptions a minimum of thirteen antigenic fractions was determined and a probable maximum of 43 fractions was estimated for these ten type 19 cultures in the ten type 19 antiserums.   Doubtless, quite different antigenic fractions would have been encountered with ten other cultures in the same antiserums.   From this it would seem that the actual number of antigenic fractions in type 19 organisms is very great and any attempt to group coliform cultures on the basis of antigenic fractions as in the genus *Salmonella*, although theoretically possible, would be wholly impracticable.

TABLE 7

Agglutination of type 13, 19 and 8 cultures in type 13 antiserums and of type 13 cultures in type 8 and 19 antiserums

| CULTURES TESTED | FOUR TYPE 13 ANTISERUM | | NINE TYPE 8 ANTISERUM | | TEN TYPE 19 ANTISERUM | |
	Per cent agglutinating	Average titer	Per cent agglutinating	Average titer	Per cent agglutinating	Average titer
22 type 13 cultures. .	23.6	1084	15.8	423	26.0	482
25 type 19 cultures.	23.0	550				
25 type 8 cultures.	11.3	380				

Type 13 cultures.   Such cultures (IMVIC $-+---$) are difficult to classify in the coliform group.   Topley and Wilson (1937) consider this type to be "non-fecal" *Escherichia*.   Parr (personal communication) considers it an atypical fecal *Escherichia*.   Stuart, Griffin and Baker (1938) and Griffin and Stuart (1940) classify this type as an intermediate, more closely related to type 19 than to type 8.   Regarding the habitat of this type, we agree with Parr since with few exceptions our 183 strains have been isolated from feces or from water, milk or soil samples containing type 19 cultures.

Four rabbits immunized with four living cultures of type 13 produced antiserums with an average titer of 11,200.   The reactions of twenty-two type 13 cultures in type 13, 8 and 19 antiserums and the reactions of twenty-five type 8 and 19 cultures in type 13 antiserums are shown in table 7.   Table 7 tends to

indicate that type 13 is more closely related to type 19 than to type 8. Six type 13 cultures agglutinating to 1250 or more reduced the homologous titer of one or another of the type 13 antiserums; eight type 19 and five type 8 cultures did not. Five type 13 cultures agglutinating to 1250 in type 8 antiserums failed to reduce the homologous titers upon adsorption. Twelve type 13 cultures agglutinated to titers of 1250 or higher in the type 19 antiserums. Four of these cultures failed to reduce the homologous titer of the serums in which they agglutinated, two reduced the homologous titer significantly while six of the type 13 cultures completely removed the homologous agglutinins from the type 19 serums. Fortunately, culture 431, a type 13 culture, removing homologous agglutinins from one of the type 19 antiserums, had been used to produce one of the type 13 antiserums. Both cultures agglutinated to the homologous titer in each antiserum, yet the type 13 culture completely removed the homologous titer from the type 19 antiserum whereas the type 19 culture failed to reduce the homologous titer of the type 13 serum. This emphasizes the importance of the reciprocal adsorption test since, on the basis of the original adsorption test, a type 13 culture would have been listed as probably identical with a type 19 organism.

It is peculiar that no type 19 culture used to adsorb the type 13 antiserums reduced the homologous titer, while eight of the type 13 cultures reduced or completely removed the homologous titer of one or another type 19 antiserum. This may indicate that if type 19 is derived in part at least from type 13, the antigenic changes consist of a loss or a covering up of a fraction of the parent antigen rather than the acquisition of new antigenic components. Whether or not this is true, there is a fairly close antigenic relationship between types 13 and 19.

Miscellaneous considerations. Five fecal strains (types 8 and 19) were antigenically identical or closely related to two fecal strains used to prepare antiserums. Two milk strains were similarly related to two milk strains used to prepare antiserums and one water strain was related to a water strain used to produce an antiserum. There was a tendency for fecal strains, especially type 19, to agglutinate more frequently, and to slightly higher

titers, in antiserums prepared from fecal strains. Apart from this, there was no correlation between the source from which the culture was isolated and agglutination.

Good correlation was found between motility and agglutination. One type 2 antiserum, however, prepared from a non-motile culture agglutinated more motile type 2 cultures than any type 2 antiserum prepared from motile cultures.

Some correlation was found between agglutination and fermentation. When the number of cultures of a fermentative group was large, the percentage of cultures agglutinating and the average titer were somewhat higher in an antiserum prepared with a culture of the same fermentative group. The correlation was not sufficiently marked to be of any practical value.

There appeared to be no correlation at all between hemolysis and agglutination in the type 19 cultures isolated from the various sources.

On the following grounds, we are inclined to believe that the per cent of cultures agglutinating and the average titer of the various types of cultures in the different types of antiserum are significant. In the early work, 103 type 2 cultures, 25 type 8 cultures and 25 type 19 cultures were tested in the nine type 2 antiserums. Similarly, 108 type 8 cultures, 25 type 2 and 25 type 19 cultures were tested in 9 group B antiserums. Also, 113 type 19, 25 type 2 and 25 type 8 cultures were tested in type 19 antiserums. Since the 25 type-heterologous cultures seemed small in comparison to more than a hundred type-homologous cultures, an additional 25 type-heterologous cultures, selected as previously described, were tested with all three kinds of antiserums. In none of the six groups of heterologous tests did the per cent of cultures agglutinating in the second set of 25 differ from the first set by more than 5. Moreover, the average titer of the second set of 25 did not differ from the first by more than 100 in any instance.

ANTIGENIC RELATIONSHIPS IN THE PARACOLON GROUP

Trawinski (1924) isolated 91 strains of paracolon organisms, a few from normal individuals, the remainder from cases of gastro-enteritis. All produced indole, formed acid and gas in glucose,

maltose and mannitol and a majority were motile. A high degree of correlation was found between fermentative reactions and agglutination.

Fothergill (1929) studied 32 cultures isolated from infants with acute diarrhea. All produced acid and gas in glucose, maltose and mannitol, many fermented lactose late, some fermented sucrose, some salicin and all were nonmotile. Unfortunately, indole production was not tested. Some correlation was found between fermentation and agglutination.

Sandiford (1935) investigated 41 paracolon cultures isolated from dysenteric stools. All the strains produced acid and gas in glucose and nearly all formed indole. The majority of the cultures were late lactose fermenters and many of these formed acid only. The 41 cultures were tested in immune serums against 9 of the cultures. The author concludes that this group of organisms is serologically diverse.

Sandiford (1935), Dudgeon and Pulvertaft (1927), Kriebel (1936) and others have found little or no reaction between paracolon cultures and typhoid and paratyphoid antiserums.

There is a recent tendency to use the term "paracolon" to designate any coliform-like organisms fermenting lactose slowly or with the formation of acid only. For these Stuart, Mickle and Borman (in press) have suggested the term "aberrant coliforms." These authors recognize five groups: pseudomicro-aerogenic, cultures producing acid and gas more readily at 20°C. than at 37°C.; micro-aerogenic, cultures requiring at least three days for the production of acid and twenty per cent gas; anaerogenic, cultures producing only acid in lactose; papillae-forming, cultures with the characteristics of *coli-mutabile*, and non-lactose-fermenting coliforms. However, in this report the term "paracolon" will be used only for aberrant coliforms found in association with pathologic conditions.

In the present investigation, 58 paracolon cultures were isolated from 58 patients and food handlers involved in a gastro-intestinal outbreak. To these cultures were added 12 paracolon cultures received from Dr. Anna Dulaney, 4 from Dr. Robert Pike and one carried in stock in this laboratory for 17 years. The 75

cultures were grouped according to their IMVIC reactions as *Aerobacter* (14), intermediate (7) or *Escherichia* (54) strains. They were further subdivided into fermentative groups according to their reactions in glucose, lactose, sucrose, salicin, maltose and mannitol. The IMVIC and fermentation reactions of each culture were determined at least three times. The cultures were further divided according to lactose fermentation and growth on eosin methylene-blue agar as shown in table 8.

The practice of assigning to the coliform group certain strains of organisms which fail to produce gas in lactose or in any other carbohydrate or do not ferment lactose, is not without precedent (Topley and Wilson (1937), Fothergill (1929), Sandiford (1935)

TABLE 8

Distribution of the paracolon cultures according to biochemical and colonial characteristics

	MICRO-AEROGENIC	ANAERO-GENIC	PAPILLATE	NON-LACTOSE	TOTAL
Aerobacter	7	3	0	4	14
Intermediates......	2	2	2	1	7
Escherichia	1	7	42	4	54

and others). Stuart, Griffin and Baker (1938) found that several coliform strains isolated as anaerogenic, in the course of several months produced gas in lactose and that occasionally aerogenic cultures lost their ability to produce gas. It will be shown later that a typical coliform strain, 143, produced a non-lactose-fermenting variant antigenically identical with the parent strain, and one strain 4411 producing acid and gas in lactose proved to be antigenically identical with another strain 7111 which did not ferment lactose.

It is becoming increasingly evident from investigations on coliform organisms in general that if the paracolon group of organisms is to be classed with the coliform bacteria, the rapid production of acid and gas in lactose or even the fermentation of lactose is in the last analysis no longer a criterion of a coliform organism. We wish to emphasize, as have Sandiford (1935) and Dudgeon (1926),

the importance of the indole test in differentiating slow or non-lactose-fermenting *Escherichia*, particularly from *Salmonella*.

Twelve of the 54 paracolon *Escherichia* cultures were selected on the basis of fermentation reactions and motility for the production of immune serums. Agglutination and adsorption tests on 8 of the antiserums were done at 37°C. In the case of 4 antiserums prepared from nonmotile or weakly motile cultures, tests were done at 55°C.

The 75 paracolon cultures were tested for agglutination in the twelve antiserums in the manner previously described. Of the 21 aberrant *Aerobacter* and intermediate cultures 7.2 per cent agglutinated to an average titer of 263. One intermediate (type 8) culture agglutinated to the homologous titer of one of the antiserums but failed to reduce the homologous titer when used to adsorb the serum. One *Aerobacter* (type 5) culture agglutinated to the homologous titer of another antiserum and reduced the homologous titer upon adsorption. No other *Aerobacter* or intermediate culture agglutinated in the antiserums beyond a dilution of 250.

Of the 54 paracolon *Escherichia* cultures 13.7 per cent agglutinated in the paracolon *Escherichia* antiserums to an average titer of 1200. This again demonstrates an antigenic specificity within the three sections of the coliform group, regardless of whether the cultures concerned are typical or aberrant. In view of the high percentage (38.4) of typical *Escherichia* agglutinating in typical *Escherichia* antiserums, 13.7 per cent of the paracolon *Escherichia* agglutinating in their antiserums is surprisingly low. As previously pointed out, we believe the number of cultures and antiserums used was sufficient to give an adequate picture of antigenic relationships in the paracolon group. Moreover, Sandiford (1935), working in Egypt, found that 13.3 per cent of 41 paracolon cultures isolated from pathologic feces agglutinated in 9 paracolon antiserums.

In general there was definite correlation between fermentative grouping and agglutination. In table 9 are shown all of the 54 *Escherichia* cultures which agglutinated in two antiserums prepared from cultures having the same carbohydrate reactions but

showing different degrees of motility. All the cultures aggluti-
nating to the homologous titer in antiserum 111 possessed the
same degree of motility and the same carbohydrate reactions.
Cultures agglutinating to low titer did not correlate with culture
111 either in motility or carbohydrate reactions. Moreover, any
one of the cultures agglutinating to the homologous titer of the
serum was capable of completely adsorbing agglutinins against

TABLE 9

Biochemical and serological reactions of selected paracolon cultures

CULTURES	FERMENTATION						MOTILITY	TITER IN ANTISERUM 111	TITER IN ANTISERUM 2711
111	+	+	+	−	+	+	+−	3750	−
2511	+	+	+	−	+	+	+−	3750	50
2711	+	+	+	−	+	+	++++	−	1250
2811	+	+	+	−	+	+	++++	−	1250
2911	+	+	+	−	+	+	+−	3750	−
3011	+	+	+	−	+	+	++++	−	1250
3111	+	+	+	−	+	+	++++	−	1250
3211	+	+	+	−	+	+	+−	3750	−
3311	+	+	+	−	+	+	+−	3750	−
3511	+	+	A	−	+	+	+−	3750	−
5311	+	A	−	−	+	+	++	50	−
5411	+	+	−	+	+	+	−	−	250
5511	+	+	+	+	+	+	−	250	−
6611	+	+	+	+	+	+	+++	50	−

Fermentation: glucose, lactose, sucrose, salicin, maltose and mannitol.

+, acid and gas; A, acid; +−, only one motile form in from 5 to 25 microscopic
fields; ++++, practically every organism motile in one microscopic field.

all other cultures including the homologous. A similar situation
occurs in cultures agglutinating in antiserum 2711.

With other fermentative groups there was a marked tendency
for cultures of a group to agglutinate more frequently, and to
higher titers, in a serum prepared from an organism having the
same carbohydrate reactions. Nevertheless, exceptions were suf-
ficiently numerous to invalidate the fermentation reactions as a
final criterion for grouping the paracolon bacteria.

The adsorption just mentioned and others showed that 23 of the
54 paracolon *Escherichia*, exclusive of the cultures used to produce

the immune serums, were capable of completely removing the homologous agglutinins from one or another of 9 of the antiserums. Seventeen cultures were able to reduce the homologous agglutinins. These findings are in sharp contrast to the results obtained with the typical coliform cultures, wherein of 324 cultures tested in 28 antiserums, only 12 cultures were found capable of completely adsorbing homologous agglutinins from any of the antiserums. No attempt was made to determine the relative number of antigenic fractions in the paracolon cultures but from a study of cross agglutination and adsorption tests, antigenic fractions appear to be quite numerous.

Rapid-fermenting variants isolated from 18 of the papillae-forming *Escherichia* cultures and their slow-lactose-fermenting parents were studied. The IMVIC and fermentative reactions were determined and the cultures tested in the 12 paracolon antiserums. In several instances where the rapid-fermenting variants and parent cultures agglutinated to high titers in one or more serums, the serums were adsorbed with both the slow and rapid strains and tested for the removal of agglutinins for each strain. From the biochemical reactions, direct agglutination and the adsorption tests, it was evident that in the 18 cultures studied the rapid-lactose-fermenting variant was biochemically (except for the speed of fermentation in lactose and occasionally in sucrose and salicin) and serologically identical with the late-fermenting parent culture.

Two cultures used in this work are especially interesting. One, culture 4411 (slow-lactose-fermenting), was isolated in 1922 from an acute case of gastro-enteritis; the other, culture 7111 (non-lactose-fermenting), was isolated in 1939 from an apparently normal food handler. Reciprocal adsorption revealed only a quantitative difference between the two strains. One or two adsorptions with culture 4411 removed all the agglutinins from antiserum 7111 whereas three adsorptions with culture 7111 were necessary to remove all the agglutinins from antiserum 4411.

Fifty paracolon cultures, including representative numbers of *Aerobacter*, intermediates and *Escherichia*, were tested in the nine type 2, nine type 8 and ten type 19 antiserums previously men-

tioned and in two additional type 19 antiserums. Of the 50 para-colon cultures only 3.4 per cent agglutinated in the 30 antiserums prepared from typical coliform cultures and but 4.5 per cent of the paracolon *Escherichia* agglutinated in the twelve type 19 anti-serums. One paracolon *Escherichia* culture (1811) agglutinated to the homologous titer in one of the typical type 19 antiserums prepared from strain T41. An antiserum was available for cul-ture 1811 and reciprocal adsorptions showed the two cultures, one a typical type 19 and the other a slow lactose fermenter, to be antigenically identical.

The percentages of typical type 2, 8 and 19 cultures agglutinat-ing in the 12 paracolon antiserums were also low. No culture agglutinated to a sufficiently high titer to warrant adsorption. Such results were not anticipated and greatly confuse the sero-logical relationship between the typical coliform organisms and the paracolon group as well as the origin of the paracolon or-ganisms.

ANTIGENIC RELATIONSHIPS BETWEEN BIOCHEMICALLY DIFFERENT VARIANTS OF SINGLE CULTURES

There have been numerous reports of variations in the bio-chemical reactions of practically all the well-known groups or species of bacteria. In some instances the cultures as a whole changed while in others a more or less stable variant was isolated from the parent culture. It is unfortunate that, in the latter instances, few investigators attempted to determine the antigenic relationships of variant and parent culture. Jordan (1920), Maver (1931) and Havens and Irwin (1932), working with non-coliform cultures, isolated stable variants differing in their bio-chemical reactions from the parent. In every case marked anti-genic differences were found between variant and parent cultures.

Working with a coliform culture Smith (1928) obtained a non-mucoid variant from a mucoid strain. The nonmucoid variant agglutinated to high titers in antiserums against itself and the mucoid parent, while the mucoid strain agglutinated only in its homologous antiserum and then only to a low titer. This varia-tion was probably due to interference with agglutination by the

capsular substance of the mucoid strain rather than to an alteration in the species-specific antigenic components.   Sievers (1937) found that an anaerogenic variant isolated from a gas-producing coliform culture had no antigenic constituent in common with the parent culture.   Minkewitsch, Rabinowitsch and Joffe (1936) and Parr (1938) found that citrate-negative variants of citrate-positive cultures were either antigenically identical with or closely related to the parent culture.   From a *Shigella flexneri* culture Smith (in press) isolated a gas-producing variant that was serologically identical with the parent culture.

In this present work on the coliform group several variants differing biochemically from the parent cultures were isolated and the serological reactions of parent and variant studied.

Culture 4411 (type 19, IMVIC $+ + - - -$) isolated from an acute case of gastro-enteritis in a patient in the Mont Alto Sanitorium in Pennsylvania in November 1922, was sent to this laboratory as a paratyphoid organism.   The culture had the characteristics of *Escherichia coli-mutabile*, producing acid and gas in lactose in 3 or 4 days.   A rapidly fermenting strain, "Black," was isolated on the first plating on eosin methylene-blue agar. The parent strain was labeled "White."   Single cell isolations were made from both the Black and White strains in December 1922 and for about one year attempts were made to produce a non-fermenting lactose variant from the White strain.   The organism was cultivated on agar containing various disinfectants, dyes and electrolytes.   Broth cultures were heated to 55°C. and samples transferred to lactose broth after various periods of heating.   Both agar slant and broth cultures were aged at 37, 20 and 5°C.   None of these methods produced a non-fermenting variant or influenced the speed of lactose fermentation significantly.

A selection method gave the best results.   Broth cultures were streaked on eosin methylene-blue agar plates which were incubated in a moist chamber for 10 to 14 days.   Several white colonies with the least number of black papillae were transferred to lactose.   That transplant which was the last to ferment was plated and treated as described.   After a number of such selec-

tions cultures were obtained which required 8 to 11 days to produce acid and gas. There appeared to be, however, no hope of securing a non-fermenting strain by this method since strains which required several days to ferment lactose sooner or later always reverted to the characteristics of the parent strain, that is, requiring only 3 to 4 days for the production of acid and gas in lactose.

The Black and White strains were used every year in classes in bacteriology to demonstrate the nature of *Escherichia coli-mutabile*. In addition, students doing special problems often used this organism in attempts to alter its characteristics. Up to and including 1938, no appreciable changes in the Black and White strains occurred. In 1939 (17 years after isolation), the first transplant of the Black strain produced acid and gas in lactose in one day whereas the White strain required 7 days. When plated on eosin methylene-blue agar the papillae forming in the white colonies varied from reddish brown to pink. Selection of colonies with the least number and lightest colored papillae after several months resulted in the first real variant, a culture producing only acid in lactose. The anaerogenic or "Acid" strain has remained stable for eight months. All biochemical reactions of the variants were identical with the parent except the fermentation of lactose and salicin.

Rabbits were immunized with living cultures of the Black, White and Acid strains. Reciprocal adsorptions of the three antiserums with all three strains showed that the Black and Acid variants were antigenically identical with the parent strain.

Culture 1 (type 2, IMVIC $- - + + +$) was transplanted every 48 hours over a period of a year in feeding experiments on *Colpoda* (Kidder and Stuart, 1939). At the end of this time two types of colonies were noted in a plating of a suspension of *Colpoda* and culture 1. One type was consistent with culture 1 and the other was a smaller, slightly mucoid colony with a small gray center. Both types of colonies were transferred to the IMVIC and several carbohydrate mediums. One type of colony gave biochemical reactions identical with culture 1. The other type gave the same biochemical reactions except that the Voges-Proskauer reaction

and growth on citrate agar were weak and no gas was produced in any carbohydrate.   Reciprocal adsorptions showed that culture 1 and the anaerogenic variant were antigenically identical.

Culture 143 (type 6, IMVIC $- - + -$A) isolated from milk, was transplanted at 4 to 5 month intervals to fresh agar slants and tested in the IMVIC mediums.   In two years the culture had shifted through type 7 to type 13.   On plating, in an attempt to reëstablish the type, a variant failing to ferment lactose was obtained.   Except for the fermentation of lactose, the strains were biochemically identical and reciprocal adsorptions showed that the lactose-fermenting parent and the non-fermenting variant were antigenically identical.

Culture 2761 (type 19, IMVIC $+ + - - -$) isolated from normal human feces was found after about 6 months in stock to have shifted to type 16 ($+ + - + -$).   The culture was plated on agar and 50 colonies were fished to citrate agar.   Several colonies failed to grow on citrate after three weeks at 20 or 37°C.   After several transplants of the citrate-negative strain had failed to grow on citrate agar, rabbits were immunized with living cultures of the negative strain and the positive variant.   Reciprocal adsorptions showed the variant to be antigenically identical with the parent strain.   Parent and variant strains have been constant in regard to growth on citrate for nine months.

Culture 1511, isolated during the epidemic previously described produced acid but no gas from glucose, maltose and mannitol in 24 hours and from lactose and sucrose in from 4 to 9 days (table 10).   Salicin was not fermented.   The culture was non-motile and generally indole-negative although on a few occasions a weakly positive indole test was obtained.   Reciprocal cross agglutination and adsorption tests of 1511 and a known *Shigella sonnei* culture in 1511 and *S. sonnei* antiserums showed that culture 1511 was antigenically identical with *Shigella sonnei*.

On one occasion, when indole positive, 1511 was plated on standard agar.   There appeared on the plates in about equal proportions two types of colonies: one translucent, the other opaque (Compton, 1932).   Culture 1511T (translucent) and 1511OP (opaque) were plated on agar and each type isolated and replated

three times. Culture 1511T continued to throw both types of colonies but the proportion of the opaque type rapidly decreased. Culture 1511OP on the other hand bred true and has remained constant for 8 months. Culture 1511T was biochemically and serologically identical with 1511. Culture 1511OP fermented the same carbohydrates as the parent strain except that acid and gas were produced in glucose, maltose and mannitol in 24 hours and in lactose and sucrose in 2 to 3 days (table 10). It was consistently indole-positive and motile although an average of 25 fields had to be examined before a motile form was found. Culture 1511OP agglutinated in antiserum 1511 but failed to reduce the

TABLE 10

Biochemical reactions of culture 1511 and related cultures

CULTURES	MOTILITY	INDOLE	GLUCOSE	LACTOSE	SUCROSE	SALICIN	MALTOSE	MANNITOL
Sonnei	−	−	A	A_7	A_4	−	A	A
1511	−	−	A	A_9	A_4	−	A	A
1511OP	+−	+	+	+	$+_2$	−	+	+
1516	+−	+	+	$+_6$	$+_4$	−	+	+
111	+−	+	+	$+_3$	$+_3$	−	+	+
112	+−	+	+	+	$+_4$	−	+	+

+−, only an occasional motile form in broth cultures; +, acid and gas; A, acid; 7, 4 etc., days required to produce specified result.

homologous titer upon adsorption (table 11). It did, however, agglutinate to homologous titer and completely adsorb all agglutinins from antiserum 111 prepared from a papillae-forming coliform. The biochemical reactions and motility of culture 111 were identical with 1511OP (table 11). Culture 1511 has on two other occasions thrown gas-producing variants biochemically and serologically identical with 1511OP.

On one plating of 1511 there appeared on several colonies granular outgrowths which were isolated and labeled 1516. This culture was biochemically the same as 1511OP except that gas was produced in lactose and sucrose in 7 to 9 days. Culture 1516 agglutinated to the homologous titer of 1511 antiserum but failed to reduce the homologous titer upon adsorption. It agglutinated

to titer and completely removed the homologous agglutinins from antiserum 111 (table 11). An antiserum was prepared from culture 112, a rapid lactose-fermenting variant of 111, to determine the antigenic relationships of 1511 and its variants to a culture which, if one lacked knowledge of its origin, must be classed as a perfectly typical strain of *Escherichia coli*.

The antigenic structure of the variants of 1511, unlike the variants of other cultures reported herein, differs markedly from

TABLE 11

Serological reactions of culture 1511 and related cultures

TESTED WITH CULTURE	ANTISERUM 1511				ANTISERUM 1511OP				
	Unadsorbed	Adsorbed with:			Unadsorbed	Adsorbed with:			
		1511	1511OP	1516		1511	1511OP	1516	111
1511	5120	—	5120	5120	320	—	80	40	80
1511OP	320	—	—		10240	10240	—	80	—
1516	10240	—		—	10240	10240	—	40	—
111	320				10240	10240	—	80	—

TESTED WITH CULTURE	ANTISERUM 111					ANTISERUM 112					
	Unadsorbed	Adsorbed with:				Unadsorbed	Adsorbed with:				
		1511	1511OP	1516	112		1511	1511OP	1516	111	112
1511	320	—	—	—		640	20	20	20	40	40
1511OP	10240	10240	—	—		20480	20480	—	—	—	—
1516	10240	10240	—	—		20480	20480	—	—	—	—
111	10240	10240	—	—	—	40960	40960	—	—	—	—
112	20480				—	40960	40960	—	—	—	—

the parent culture. A more detailed analysis of flagellar, somatic and phi antigens and their qualitative and quantitative differences is now being made and further discussion of this aspect of the problem will be reserved for a subsequent report.

DISCUSSION

In the work on coliform bacteria, in this laboratory and in the Connecticut State Laboratories at Hartford, Connecticut, 10,752 cultures have been isolated from water, soil, milk, dust, hides, cereals and feces. These cultures, with the exception of a rela-

tively few irregulars, have fitted into the 19 types as previously proposed on the basis of their IMVIC reactions. It must be admitted that the types within the groups and even the groups are not wholly constant in their biochemical reactions. Shifts in type occur in orderly sequence within any group, with occasional shifts from one group to another (Stuart, Griffin and Baker, 1938) and (Griffin and Stuart, 1940). In the great majority of cases, particularly in the basic types 2, 8 and 19, cultures are stable. Despite these occasional fluctuations some bacteriologists contend that generic distinction should be made between the three groups. Others place the intermediates in the genus *Escherichia*, while some would recognize but a single genus for the three basic types or the three groups as a whole. Considering the occasional instability of strains, strict lines of antigenic demarcation were not anticipated. In the early part of the work, where single cultures of several types were used for the production of immune serums and several cultures of the various types tested in the immune serums, few, if any, lines of demarcation could be drawn between types or even groups. When, on the other hand, several cultures of a basic type were used to produce immune serums and a large number of type-homologous and type-heterologous cultures tested in the serums, the average titer of the type-homologous cultures was always significantly higher than the average titer of the heterologous types. Thus, the serological evidence confirms the biochemical evidence in showing a certain degree of fixity of the basic types. This might be said of the types within the group, since types 1, 5 and 13 gave some evidence of antigenic homogeneity. We are satisfied, however, that in general practice any attempt to differentiate biochemically or antigenically beyond the basic type within the group would be defeated by a vast number of intergrades.

Any comment on an evolutionary tendency within the coliform group as a whole must be made with considerable reservation. Nevertheless, certain facts in the present investigation are at least worth mentioning in this respect. The coliform group probably originated from some primitive type or types of bacteria. Organisms in the genus *Erwinia* resembling the coliform or-

ganisms, particularly the *Aerobacter* and type 1 cultures, may be
taken as a point of departure.   If permanent implantation of the
plant forms occurred or occurs in man, the new environment
might cause some degree of specialization.   If the known *Erwinia*
and plant coliforms we have worked with are indicative of the
genus as a whole we wonder whether *Erwinia* are not coliform
organisms with plants for a host, *Escherichia*, coliforms with
animals for a host and *Aerobacter* and intermediates, coliform
organisms which have lost their pathogenicity for plants without
yet attaining permanent implantation in animals.

There is some serological evidence to show antigenic specializa-
tion in the coliform group.   In no instance was any type 2 culture
found sufficiently related to the type 2 (*Aerobacter*) cultures used
for immunizing to adsorb a significant amount of the homologous
agglutinins.   With the type 8 (intermediate) cultures, 9 instances
were found where cultures tested were identical with or anti-
genically related to the cultures used to produce the immune
serums.   In the type 19 cultures 45 such instances were found.
Specialization in the group is further substantiated by the fact
that type 2 cultures which possess great antigenic diversity are
limited in community of antigenic fractions whereas in type 19
cultures, antigenically less diversified, common antigenic frac-
tions are quite numerous.   To continue the comparison, in the
Salmonella and *Shigella* genera, community of antigenic fractions
increases until in the latter genus with few exceptions every
species is serologically related to every other species.

Any classification of bacteria must take into account two quite
different view points, the technical and the academic.   Two sepa-
rate systems are impractical, therefore compromises must be
made.   In classifying the coliform organisms one is immediately
faced with a dilemma.   To overlook the differences between some
Aerobacter cultures with their abundant growth on agar, their
ability to grow on citrate, to produce acetyl-methyl-carbinol, to
liquefy gelatin and to ferment such carbohydrates as sucrose,
salicin, dulcitol and adonitol and some *Escherichia* cultures with
their moderate growth on agar, their inability to grow on citrate
agar or to produce acetyl-methyl-carbinol, or to liquefy gelatin

or to ferment any of the carbohydrates specified, is impossible. On the other hand, to recognize all the cultural, biochemical and antigenic intergrades between the two types is equally impossible. The American Committee on Classification recognizes two species, *aerogenes* and *cloacae*, in the genus *Aerobacter* and two species *freundii* and *coli* in the genus *Escherichia.* The present work on the biochemical and serological aspects of the problem compels us to take a few minor exceptions to the Committee's point of view. There appears to be no serological evidence, and the biochemical evidence seems to us inadequate, to support the differentiation between *aerogenes* and *cloacae* (Griffin and Stuart, 1940). While we agree that some distinction should be made between the intermediates and *Escherichia*, we cannot support the theory that the intermediates as represented by type 8 (*freundii*) are more closely related to *Escherichia* than to *Aerobacter*, either biochemically or antigenically or on the basis of habitat (Griffin and Stuart, 1940). An antigenic specificity in the basic types of groups A, B and C has been shown in this work. Group antigenic specificity, however, loses some of its force when it is noted, for example, that the average titer of type 19 cultures in type 19 antiserums is approximately only three dilutions higher than the average titer of type 2 cultures in the same antiserums. For this reason, and also because types of one group do shift occasionally to another group, we suggest a single genus presumably *Escherichia*, with three species, *aerogenes*, *freundii* and *coli*.

Distinguished from the typical coliforms are the paracolon bacteria, a group of coliform-like organisms producing acid and gas slowly or acid only in lactose. Many of the strains in this group producing acid and gas slowly throw rapid-lactose-fermenting variants, and some acid-producing strains throw gas-producing variants. Some of the strains in this group are thought to be definitely pathogenic. While pathogenicity of others is very doubtful, they are almost always associated with gastro-intestinal disturbances in man. If these organisms are even mildly pathogenic, or if they are entirely nonpathogenic but are associated with pathologic conditions in man, the significance of the group

as a whole should not be confused by including in it all the aberrant coliform types.

From natural sources, water, soils and cereals, large numbers of organisms have been isolated which produce acid and gas slowly or acid only in lactose (Stuart, Griffin and Baker, 1938) and (Griffin and Stuart, 1940). From fifty samples of soil, for example, taken from well-posted ground surrounding a reservoir, 107 coliform cultures were isolated, of which 103 were slow-lactose-fermenters. It is difficult to believe that aberrant coliforms isolated from natural sources and those isolated from man during gastro-intestinal epidemics are, generally speaking, identical. There is some evidence to show that they are not. A review of the literature and our work on several hundred aberrant coliform cultures show that practically all such cultures isolated from natural sources are *Aerobacter* and intermediates while the large majority from man are *Escherichia*. The paracolon organisms have been considered intermediate between the typical coliforms and the *Salmonella* (Kriebel, 1936) or *Shigella* groups (Wilson, 1929), (Parr, 1939) but it would be difficult to place aberrant coliforms from nature in the same position.

It is possible that the coliform group, from *Aerobacter* through the intermediates to *Escherichia,* is a succession of degrees of specialization and that strains aberrant with respect to lactose-fermenting ability exist at either end of the succession. The aberrant coliforms from unpolluted natural sources might then be considered more primitive organisms leading up to the typical coliform group whereas paracolon *Escherichia* organisms as described in this paper are more specialized, tending toward the *Salmonella* or *Shigella* groups. There is good evidence to support the succession theory within the typical coliform group (Parr, 1939), (Stuart, Griffin and Baker, 1938), (Griffin and Stuart, 1940) some evidence for the transition from primitive to typical coliform (Stuart, Griffin and Baker, 1938) (Griffin and Stuart, 1940), but little evidence to support the transition from coliform to the pathogenic forms. Our first assumption, in agreement with Parr (1934) and Dulaney and Smith (1939), that the aberrant coliforms associated with epidemics in man might result

from the effect of the pathologic condition upon the normal flora of the intestine, was made less tenable by the present work.   If the aberrant types from man are merely altered normal coliforms, it is difficult to account for the lack of antigenic relationship between the aberrant and the normal forms, particularly since, in no case, was an antigenic difference proved between the slow and rapid lactose-fermenting strains of any papillae forming culture.

The gas-producing variants of *Shigella sonnei* reported herein and the gas-producing variant of *Shigella flexneri* reported by Smith (in press) suggest another possibility, namely, that certain slow lactose fermenters are variants of the etiological agent in the epidemic.   It is true that the instances reported herein and by Smith may be rare exceptions but other considerations lead us to suspect they are not.   The relatively close, in some cases complete, antigenic relationship between certain strains of *Shigella sonnei* and *Shigella flexneri* and certain coliform strains shown by Mackie (1939) and the fact that in gastro-intestinal epidemics the slow-lactose-fermenters are most abundant in feces after the etiological agent has disappeared, tend to support the suggestion that certain slow lactose fermenters may be variants of the etiological agent rather than variants of typical coliforms.

CONCLUSIONS

1. The serological findings appear to confirm the biochemical grouping previously proposed for the coliform organisms.

2. There is evidence of antigenic differences among the three groups.   The *Aerobacter* group is greatly diversified, with but few antigenic fractions, while *Escherichia* is less diversified, with numerous antigenic fractions.   The intermediates appear to be more diversified than *Aerobacter* but less than *Escherichia*.

3. It is suggested that the organisms now known as the coliform bacteria be placed in a single genus, presumably *Escherichia*, with three species—*aerogenes, freundii* and *coli*.

4. Antigenically, the paracolon group of bacteria appears to be less closely related to typical *Escherichia* than are the three sections of typical coliforms to each other.

5. There is, in general, fair correlation between agglutination and fermentation reactions but not sufficient to permit the classification of the paracolon group on the basis of their biochemical reactions.

6. The use of the term "paracolon" should be limited to papillae-forming, anaerogenic and non-lactose-fermenting *Escherichia*-like organisms associated with gastro-intestinal disturbances, and its use should be discontinued as soon as it becomes possible to apply more descriptive terms to the various aberrant coliforms comprising it.

7. A review of the literature and the present work show that variants isolated from single cultures and differing biochemically from the parent may be antigenically identical with, related to, or entirely different from the parent culture.

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PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

JOINT MEETING, NORTH CENTRAL AND INDIANA BRANCHES AND THE SOCIETY OF ILLINOIS BACTERIOLOGISTS

NORTHWESTERN UNIVERSITY MEDICAL SCHOOL, CHICAGO, MAY 3rd and 4th, 1940

EVALUATION OF GRAM STAINING. *Eleanor P. Burton,* Coordinating Laboratory, Ill. Dept. Public Health.

Numerous modifications have been developed from Gram's original staining method. The Committee on Bacteriological Technique of the Society of American Bacteriologists made a survey of these staining procedures and today recommends the Hucker and the Kopeloff-Beerman methods. This also led to the establishment of the Commission for Certification of Biological Stains.

During the past two years, the Illinois Co-ordinating Laboratory has examined 8,000 positive and negative gonococcus smears sent to 265 laboratories of the State as check specimens for diagnostic laboratory approval.

Gram-staining ability as exhibited by these laboratories has proved on the average to be mediocre in quality.

There is little correlation between staining method used and quality of work done. Modifications fall into five groups, according to type of primary stain: Phenol, aniline, miscellaneous mordants and alkalis, aqueous solutions without mordant.

One-fifth of the laboratories in the State obtain consistently excellent results; two-fifths show neither steady improvement nor progressive failure; two-fifths have brought their technique up from an unsatisfactory to a satisfactory or excellent level. The majority of this last group have improved in becoming more proficient with the method they had always used rather than by changing from one method to another.

(Kodachrome pictures were shown illustrating various results on stained smears.)

THE USE OF SODIUM LAURYL SULPHATE LACTOSE TRYPTOSE BROTH AS A PRIMARY MEDIUM FOR DETECTION OF COLIFORM GROUP. *W. L. Mallmann and C. W. Darby,* Dept of Bacteriology, Michigan State College, East Lansing.

The bacteriostatic titres, on grampositive and negative organisms, of Aerosol, M.A., Nacconol N.R.S.F., Duponol paste W.A., Santamerse #1 and Igepon T. were determined. Nacconol N.R.F.S. and Duponol paste W.A. inhibited streptococci and *Bacillus megatherium* in dilution of 1:30,000. Aerosol M.A. and Igepon T. showed no inhibitory effect on gram-positive organisms. Action was not due to surface depressant effect as all of the wetting agents had approximately the same value. By the use of growth curves it was found that in a concentration of 1 to 10,000, neither Duponol paste W.A. or Nacconol N.R.F.S. had

any inhibitory action on coliform organisms. Duponol paste W.A. was added to the tryptose lactose broth reported by the authors (J.A. Water Works Assoc. **31**: 689, 1939) in concentration of 1 to 10,000. This medium when used in parallel with standard lactose broth on tap waters showed that in standard lactose broth, 238 primary tubes were positive but only 4 confirmed, while only 3 primary tubes in lauryl sulphate tryptose broth were positive and all of them confirmed. On raw waters the positive primary tubes in lauryl sulphate tryptose broth which failed to confirm were found by other technic to contain coliform organisms.

BACTERIAL-FUNGUS   ANTAGONISMS. *C. L. Porter*, Division of Botany, Purdue University.

Fungi are profoundly affected in their growth, development, metabolic activities, and pathogenicity by the presence of other organisms. The phenomena of inhibition and accelerated development have both been observed. Phenomena associated with mixed cultures are difficult to study, but such investigations are profitable because mixed cultures occur often in nature and frequently account for peculiarities of growth and the eccentricities of infection.

Associations of species of filamentous fungi have been noted by many students, but the effects of such associations upon the fungi concerned are not as marked, usually, as are the effects of bacteria or their products upon fungus development. Bacterial organisms vary considerably in their power to affect the growth of filamentous fungi.

Media which have supported certain bacterial cultures and media after prolonged sterilization, exhibit marked inhibitory tendencies to further growth of fungi on such media. All bacterial species are not equally effective in producing such inhibition. Certain bacteria are apparently incapable of producing any measurable effects.

PRECIPITIN PRODUCTION IN THE ALBINO RAT. *Guy P. Youmans and Charlotte A. Colwell*, Department of Bacteriology and Department of Research Bacteriology, Northwestern University Medical School, Chicago, Illinois.

Albino rats were given repeated injections of sheep serum. Rapidly flocculating antiserums having low antigen dilution titers and relatively high antiserum dilution titers were obtained. Constant antiserum optimal ratios were as low as 1:0.2.

THE INFLUENCE OF PANTOTHENIC ACID, NICOTINIC ACID, BIOTIN CONCENTRATES AND OTHER SUBSTANCES ON THE GROWTH OF PROTEUS IN A CHEMICALLY DEFINED MEDIUM. *Michael J. Pelczar, Jr., and J. R. Porter*, Department of Bacteriology, College of Medicine, State University of Iowa, Iowa City.

Investigations in this laboratory have shown that most *Proteus* species with the exception of *Proteus morganii* are capable of continued growth in a simple synthetic medium containing inorganic salts, glucose and nicotinic acid. *Proteus morganii* requires, in addition to these constituents, pantothenic acid and several amino acids.

The efficacy of these synthetic media, with regard to the quantity of growth produced, was compared to that of meat infusion broth. The organisms were cultured in 50 ml. volumes of the media, and the amount of growth was measured in terms of bacterial nitrogen by use of a micro-Kjeldahl technique.

The resulting values indicate that meat infusion broth is far superior to the synthetic media.

In an attempt to procure a medium of synthetic composition which would be capable of yielding a quantity of growth approaching that obtainable in meat infusion broth, the basic synthetic media were supplemented by the addition of "biotin" (prepared from egg yolk), Bios IIB (a Standard Brands product), inositol, glutamine, B-alanine and several other substances in the category of growth factors. The resulting values for bacterial nitrogen were not equal to those obtained when the same organism was cultivated in meat infusion broth.

THE EFFECT OF BIOTIN CONCENTRATES ON THE GROWTH OF STAPHYLOCOCCUS AUREUS. *J. R. Porter and Michael J. Pelczar, Jr.*, Department of Bacteriology, College of Medicine, State University of Iowa, Iowa City.

It has been observed that certain strains of *Staphylococcus aureus* are unable to grow on continued subculture in the chemically defined medium of Gladstone. In an effort to find the substance or substances which would supply the needs of these strains, several growth-accessory substances were tested. When the medium was supplemented with minute amounts of a biotin concentrate prepared from Armour's Cloverbloom dried egg yolks (procedure of Kögl and Tönnis), it supported growth of the above strains on continued subculture.

The effect of biotin on the growth of several other strains of *Staphylococcus aureus* has been studied. Preliminary experiments indicate that the organisms may be allocated to three groups on the basis of their response to biotin: (1) those which grow on continued subculture in the chemically defined medium of Gladstone and are not influenced by biotin, (2) those which will continue to grow in Gladstone's medium but are stimulated by the addition of biotin, and (3) those which will not grow on continued subculture unless biotin is present.

EFFECT OF AMMONIA ON THE GERMICIDAL EFFICIENCY OF CHLORINE IN NEUTRAL SOLUTION. *George R. Weber, Richard Bender and Max Levine*, Iowa State College, Ames, Iowa.

It was observed that the time necessary to kill 99% of exposed spores of *Bacillus metiens* increased with increasing amounts of ammonia but was neither a direct function of the available chlorine added nor of the residual chlorine.

Residual chlorine, in the presence of ammonia, was found not to be a direct function of the chlorine added but rose to a maximum, then dropped to a minimum of practically zero when the ratio of chlorine to ammonia was approximately 7 to 1, while further additions of chlorine resulted in corresponding increases in chlorine residuals.

With water buffered at pH 7.0 containing 10 p.p.m. ammonia it was found that approximately the same residuals (22.5, 24.2 and 24.9 p.p.m. available chlorine) were obtained on addition of 95.5, 61.5 and 28.0 p.p.m. chlorine, the respective killing times being 2.7, 88 and 83 minutes. In view of these results it seems probable that at a point below the minimum residual the available chlorine exists in the form of chloramines; that at the point of minimum residual probably all of the ammonia has been oxidized, and that beyond this point the residual is due to HOCl, which is particularly effective as a germicide.

THE MODERN CONCEPTION OF THE BACTERIAL QUALITY OF WATER. *John R. Baylis*, Department of Public Works, Chicago, Ill.

The author recommends the adoption of a bacterial standard more rigorous than that known as the Treasury Department Standard. Complete elimination of lactose-fermenting organisms is suggested. More than 1.0% of 10-ml. tubes or more than 10.0% of 100-ml. tubes within a month is unsatisfactory. Total count at 37° not more than 2/ml. No more than 50% of positive presumptives should confirm.

Sewage polluted water should carry 0.5–1.0 p.p.m. residual chlorine for 2 hours regardless of bacterial purity of the water. Chlorine resistant organisms should be removed regardless of amount of chlorine necessary.

EFFECT OF SEWAGE TREATMENT AND LAKE WATER DIVERSION AT CHICAGO ON SANITARY CONDITIONS OF MAIN CHANNEL AND THE ILLINOIS RIVER. *F. W. Mohlman*, Director of Laboratories, Sanitary District of Chicago.

Reduction of water drawn from Lake Michigan for dilution of sewage, from 5,000 cu. ft. per second in 1938 to 1,500 c.f.s. on Jan. 1, 1939, resulted in a great deterioration in the condition of the water in the Main Channel and the Upper Illinois River. The oxygen balance, rather than numbers of bacteria, measures the degree of pollution, as the problem relates to nuisance since the Illinois River is not used for water supply. The dissolved oxygen content showed a sudden and unprecedented lowering in 1939, and the biochemical oxygen demand greatly increased. Conditions in the summer of 1939 were the worst in 20 years. There is a marked improvement in 1940 to date (May first) because of

operation of the Southwest activated sludge plant, the largest in the world. River samples are collected hourly at stations from Lake Michigan to Peoria. About 7,000 determinations of dissolved oxygen are made per month. A temporary increase in diversion of water is needed.

THE CONCENTRATION OF A LYTIC PRINCIPLE IN SEWAGE. *Maryalys E. Klein*, Department of Research Bacteriology, Northwestern University Medical School, Chicago, Illinois.

The lytic principle in fresh sewage filtrates was concentrated by adsorption on aluminium hydroxide cream at pH 5.0 and elution in phosphate buffer of pH 7.4, and a comparison was made of the properties of this concentrated principle with those of mature broth phage.

The concentrated lytic principle was inactivated by the same physical and chemical agents that inactivate broth phage; it was specifically adsorbed by heat-killed organisms; it gave rise to the production of antibodies which inhibited its lytic activity. From this evidence it can be concluded that the lytic agent which is adsorbed from sewage by aluminium hydroxide cream is bacteriophage.

CELLULOSE-SPLITTING BACTERIA FROM HUMAN STOOLS: A PRELIMINARY REPORT. *Nell Hirschberg*, Department of Bacteriology and Public Health, University of Illinois College of Medicine, Chicago, Illinois.

Cellulose-fermenting organisms have been isolated and studied for many years. The use of older methods using cotton, filter paper, or precipitated cellulose is tedious, and therefore it was determined to try the method of Aschner using the cellulose membranes

from the growth of *Acetobacter xylinum*.

Diluted stools were spread over the surface of the membrane with a glass spreader and cultured for at least two weeks. Aschner observed colonies sunk down into the medium, and evidence of liquefaction after 48 hours, but in our experience many of the organisms took much longer to grow. At the present time we have studied 13 strains of organisms isolated from the membranes. All of these organisms eat down into the medium.

Dr. Donald S. Martin of Duke University was kind enough to check the cultures of the fungi. Four organisms were strains of *Aspergillus*, one was a strain of *Aspergillus niger*, one was a strain of *Mucor*, while 2 were strains of *Penicillium*. In addition, 2 strains of *Saccharomyces* and 3 strains of unidentified yeast-like fungi have been isolated. Further identification of these organisms is now under way.

Much more work on the cellulose splitters in human stools is indicated. While this is a preliminary report, it shows the high incidence of fungi in stools and the usefulness of a relatively little known method. Biochemical studies of the cellulose membranes have been made. Since new cellulose-splitting strains are continually being isolated at the same time, the method is being used to study the ability of the common, well-known bacteria to utilize cellulose.

THE OCCURRENCE OF DIPHTHERIA ANTITOXIN IN THE HUMAN PREGNANT MOTHER, NEWBORN INFANT, AND PLACENTA. *Joseph Liebling and Guy P. Youmans*, Department of Bacteriology Northwestern University Medical School and Obstetrical department of St. Vincent's Hospital of Chicago.

The problem concerns itself with the development of diphtheria antitoxin in the human pregnant mother, newborn infant and placenta.

A group of mothers were actively immunized with diphtheria toxoid in the latter part of their second trimester and a second group of equal number was used as a control. Diphtheria-antitoxin-titer determinations of the blood of the immunized and non-immunized mothers was obtained at monthly intervals and upon termination of their pregnancy. Schick-negative mothers showed a much more rapid response to immunization than Schick-positive mothers. Non-immunized Schick-negative mothers showed a definite rise in antitoxin level as compared with Schick-positive mothers.

Cord blood was obtained from all infants at the time of delivery. The diphtheria antitoxin level and Schick test of the infant were comparable with the findings in the mother.

The placentae of both the immunized and non-immunized mothers were extracted according to the method of McKhann, and the diphtheria antitoxin content determined. The antitoxin content of the placenta in both immunized and non-immunized mothers was very small when compared with the antitoxin content of the blood of the mother and infant at termination of pregnancy.

CULTURAL STUDIES ON GONOCOCCAL INFECTION IN WOMEN. *Lucile R. Hac*, Department of Obstetrics and Gynecology, The University of Chicago, and The Chicago Lying-in Hospital.

Cultural studies have been used as the criterion of cure in the evaluation

of certain therapies used in gonococcal infection in women. The inoculated swabs were placed in about 0.3 ml. of broth and then streaked on plates from a few minutes to three hours later. The plates were left at room temperature until they were returned to the laboratory about five hours later. Transportation required 30 to 45 minutes. No special precautions were taken to keep the material warm even in weather as cold as $-13°F$. The results obtained under these conditions confirm the results of others concerning the superiority of cultures over smears.

Cultures and smears were taken from 3 to 32 times on 216 patients; 1656 cervical specimens were compared with 1675 urethral specimens. Culture and smear examinations were both positive in 23.5 per cent of cervical and 8.1 per cent of the urethral specimens; 51.8 per cent positive cervical and 69.1 per cent positive urethral cultures had completely negative smears. Only 0.56 per cent of both positive and suspicious smears gave negative cultures. Thirty-two patients had positive cervical cultures 3 to 51 days after urethral cultures had become negative. Seven patients had positive urethral cultures 3 to 40 days before cervical cultures became positive and 7 had positive urethral cultures after cervical cultures had become negative.

Twelve patients had consistently positive cultures from 16 to 126 days after smears had become negative.

ARTIFICIAL TRANSMISSION OF MALARIA. *G. Howard Gowen*, University of Illinois College of Medicine and the Chicago Health Department.

Artificially-induced malaria fever in the treatment of neurosyphilis, and malaria in drug addicts resulting from common use of the hypodermic needle is discussed as a potential reservoir from which malaria may be spread to the general population.

Three outbreaks totalling 18 cases have been reported in which the disease may have been transmitted by anophclene mosquitoes from inoculated paretics to those infected, as a result of laxity in the isolation and terminal release of those receiving the malaria therapy. It is suggested that all institutions employing malaria therapy keep such patients in properly screened quarters during the mosquito season, give them a standard course of anti-malaria therapy after completion of treatment, and do not release such patients as free from malaria until blood smears show an absence of the malarial parasite.

Malaria in drug addicts is common in Chicago and New York City, and is found in other large cities in the United States. It is spread by common use of the hypodermic needle. Both the benign tertian (*Plasmodium vivax*) and the malignant tertian (*Plasmodium falciparum*) strains are found. In the fatal cases *Plasmodium falciparum* is the usual etiologic agent. In Chicago and New York City, respectively, from 1937 to 1939, 41 and 46 per cent of the total reported cases were drug addicts. In both cities more than 80 per cent of the deaths from malaria occur in the addicts. The control of malaria in drug addicts could be attempted, but whether such a preventive program would be feasible is open to question.

THE EFFECT OF SULFANILAMIDE ON STAPHYLOCOCCAL DEHYDROGENASES. *Z. John Ordal and H. Orin Halvorson*, Department of Bacteriology, University of Minnesota.

The effect of sulfanilamide on

staphylococcal dehydrogenases has been investigated using washed cell suspensions. The dehydrogenase activity was determined in modified Thunberg tubes in which anaerobiosis was obtained by bubbling oxygen-free nitrogen through the tube instead of removing the air by evacuation.

Sulfanilamide in low concentrations (120 p.p.m.), added directly to the Thunberg tube, had no effect on any of the dehydrogenases tested. Exposing the cells to a high concentration of sulfanilamide before testing the dehydrogenase activity was also without any effect. When a high concentration of sulfanilamide (0.7%) was added to the Thunberg tube, the dehydrogenase activity was definitely decreased. The effectiveness of sulfanilamide at pH 7.5 varied with the different substrates. With glucose dehydrogenase the inhibiting effect was found to vary with pH. At pH 7.75 and above, the effect was very marked. Below pH 7.75, the effect decreased as the pH was decreased.

Acid production in growing cultures on glucose broth was correlated with population increase. In cultures containing sulfanilamide, the lag in acid production was greater than the lag in population. This would indicate that sulfanilamide inhibits the utilization of glucose or that it alters the mode of fermentation.

THE ACTIVITY OF SULFONAMIDE DRUGS IN EXPERIMENTAL PNEUMOCOCCAL INFECTIONS. *Marjorie Fogas*, The Lilly Research Laboratories, Indianapolis, Ind., U.S.A.

In the course of chemotherapeutic assay of various drugs, we have repeatedly treated different types of pneumococcal infections with Sulfanilamide, Sulfapyridine, 4,4'diamino diphenyl sulfone, dipropionyl amino

diphenyl sulfone and Sulfathiazole respectively.

In a comparative way, insoluble drugs appear more effective than soluble drugs. In these instances the soluble drugs have been less toxic and have prolonged the life of mice considerably.

The action of Sulfanilamide in repeated doses of 5–10 mg. in such pneumococcal mice is mainly a prolongation of life as compared to untreated controls. In our five dose treatment we have been able merely to prolong the lives of mice up to 3.8 days.

On the other hand, Sulfapyridine, in a series of fifty tests using five repeated doses of 30. mg. each, cures 300 out of 500 mice with an average of 9.4 days.

4,4'diamino diphenyl sulfone is an extremely effective drug, but ten times as toxic as Sulfanilamide and produces typical sulfone reactions in doses within the toxic range. Repeated doses of 0.5 mg. per mouse give an average of 6.3 days survival.

Dipropionyl amino diphenyl sulfone is a relatively unknown drug and repeated doses of 30 mg. give an average survival of 8.7 days.

One of the most recent publicized drugs, Sulfathiazole, is very effective when given in repeated doses and compares favorably with Sulfapyridine in effectiveness.

COMMON COLD VIRUS INOCULATIONS. *H. M. Powell, A. L. Sparks, and G. H. A. Clowes*, Lilly Research Laboratories, Indianapolis, Indiana.

Six strains of cold virus have been inoculated intranasally after various intervals into human subjects, mainly during the summer months when natural cold incidence was lowest. In general, only about two thirds of such persons contract inoculation colds in

the two or three days following inoculation.

Evidence has been obtained that these strains of cold virus may be maintained for a time by intranasal passage in series in small swiss mice. Mild lesions only are to be found in such mice, including congestion and bronchial catarrh. The latter is never sufficiently intense to lead to collapse or filling up of alveoli and none of the mice die, unless a Pfeiffer-like bacillus is picked up in passage. In such cases a high percentage of deaths results.

We have attempted cold-virus passage in ferrets with the result that no noteworthy reactions were obtained with the exception of a rather frequent development of subnormal temperature.

In neither mice nor ferrets have the effects of cold virus in serial passage been sufficiently severe to use these animals to advantage in routine handling of cold virus in the laboratory.

As a continuation of these inoculation experiments, we have tried cold virus vaccine as a prophylactic in 83 adult persons and have observed some immunization against artificial colds and little or no immunization against natural colds.

PATHOGENESIS AND IMMUNITY IN EC-TROMELIA VIRUS INFECTION OF NASAL MUCOSA OF THE WHITE RAT. *H. R. Reames and F. B. Gordon*, University of Chicago.

An investigation of the immune mechanisms operating in the subclinical infection of rats with ectromelia virus introduced intranasally was used as an approach to the problem of resistance in man against those virus diseases whose portal of entry is the upper respiratory tract.

The course of infection was studied by sacrificing the rats at intervals and testing the nasal mucosa for the presence of virus by intraperitoneal inoculation into mice. In agreement with Burnet, we found that virus at first increases in amount in the nasal mucosa, then disappears, and that several such exposures to the virus render the rat immune to further inoculation.

The role of the serum antibody in this resistance was studied by neutralization tests with serums of rats taken after various types of inoculation. No evidence was found that the serum antibody was of significance. Saline extracts of nasal mucosa of immune rats did not possess detectable neutralizing ability.

Histological study of the nasal cavity of rats at intervals after inoculation revealed no differences in the tissue reaction between normal and immune rats.

The mechanism of this resistance in the rat was not elucidated, but the evidence suggests that it resides locally, in the nasal mucosa.

STUDIES WITH BCG IMMUNIZATION. *Dr. Sol Ray Rosenthal*, University of Illinois, College of Medicine, Chicago, Illinois.

BCG vaccination has been shown to exert a definite amount of protection against tuberculosis, both in animals and man. A simple, efficacious and safe method of introducing this vaccine into human beings has been described. By the multiple puncture technique, fewer organisms are used and no complications result. The pathogenesis of the multiple puncture technique and the intradermal technique, as determined by microscopic sections, has been elaborated upon. By comparison with other authors using oral, intradermal, or subcuta-

neous routes of inoculation, the precocity and duration of the tuberculin reaction has been much augmented by the multiple puncture method.

SOME BIOLOGIC CHARACTERISTICS OF OCULAR STRAINS OF STAPHYLOCOCCI. *J. H. Allen and O. N. Fellowes*, Departments of Ophthalmology and Bacteriology, College of Medicine, State University of Iowa, Iowa City, Iowa.

There have been many tests devised to determine whether a certain staphylococcus is pathogenic or not.

Such tests as the pigmentation of the colony, the ability to produce hemolysin on blood agar, the fermentation of mannitol, the reaction on crystal-violet agar, the production of coagulase, the liquefaction of gelatin, the production of toxin, and the determination of agglutinative group have been performed on 100 strains of ocular staphylococci, chosen at random.

The records of the patients' pathology and response to therapy were used as criteria of the pathogenicity of the strains. There were 54 strains from eyes with pathology and 46 strains from eyes showing no pathology. The grouping, according to the various cultural and toxigenicity tests, was determined and assessed for false positives and negatives. These corrected values in turn were compared with the agglutinative grouping performed with 4 staphylococcus antisera reciprocally absorbed. It was found that toxin production, mannitol fermentation and serological grouping were the more reliable criteria for the determination of pathogenicity.

A COMPARISON OF METHODS FOR DETERMINING GELATIN LIQUEFACTION BY STAPHYLOCOCCI. *Shirley B. Penruddocke and Edith L. Swingle*,

Department of Bacteriology, Iowa State College.

A comparative study of gelatin liquefaction by 70 cultures of staphylococci from pathogenic, non-pathogenic, and food-poisoning sources was made by means of viscosity determinations in the Ostwald viscosimeter, gelatin stabs, and Stone's gelatin agar plates.

Although the viscosimeter had been found satisfactory for measurement of gelatin liquefaction by bacilli, it proved to be unsuitable for cocci because it failed in many instances to detect slight liquefaction which was readily discerned by other methods.

Gelatin stabs were incubated at 20°C. and 37°C. At 37°C., stabs were found to be unreliable since many cultures which were negative by this method showed definite liquefaction at 20°C. On the other hand, there were no cultures which were positive at 37°C. and negative at 20°C.

A study of Stone's plates incubated at these two temperatures indicated that temperature affects gelatin liquefaction in some instances. While many of the cultures gave identical readings at both temperatures, some were positive at 37°C. but negative at 20°C. and others were positive at 20°C. but negative at 37°C.

A comparison of Stone's plates with the 20° stabs showed that all cultures positive on Stone's were positive in the stabs after 48 hours, but that they did not always show any liquefaction in 24 hours. A few cultures negative on Stone's at 37°C. were positive in stabs. Part of these were positive on Stone's at 20°C., indicating that temperature of incubation was sometimes responsible for the discrepancy. The rest might be accounted for by the differences in composition of the two media and in methods for observing changes in the gelatin.

IMMUNOLOGICAL STUDIES ON HUMAN "STORED" BLOOD PRESERVED WITH SULFANILAMIDE. *Milan Novak**, Department of Bacteriology and Immunology, University of Minnesota.

The addition of twenty milligrams of sulfanilamide per one hundred milliliter, of blood or plasma to be used for transfusions, prevents gross bacterial contamination of such products during the period of storage. Immunological studies on the fate of complement and phagocytes in such stored blood were carried out.

The amount of complement was titrated in stored citrated blood and in stored citrated blood containing sulfanilamide as a preservative. The complement remained stable for about two days, after which it deteriorated so that nearly eighty per cent destruction had taken place in twelve days and almost one hundred per cent after twenty days. There was no significant difference between blood containing sulfanilamide and that without the drug in respect to the stability of complement.

Periodic phagocytic counts made on similar samples using a stock strain of *Staphylococcus albus* revealed no adverse effect of the preservative on the leukocytes. A progressive functional impairment, however, took place in the phagocytes in all the samples so that average phagocytic counts of ninety-three, sixty, sixteen, seven and one-half per cent were observed after the first, second, third, fourth and fifth days of storage respectively. There were no significant differences in the counts made on stored blood containing sulfanilamide and blood without the preservative.

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FURTHER STUDIES IN EXPERIMENTAL SUB-ACUTE BACTERIAL ENDOCARDITIS AS A CAUSE OF DEATH. *P. F. Clark*, Department of Medical Bacteriology, University of Wisconsin, Madison.

ANIMALS IN EXPERIMENTAL TUBERCULOSIS. *A. A. Day*, Northwestern University Medical School.

Extensive research has greatly increased our knowledge of tuberculosis and at the same time has raised unsuspected problems concerning which there exist uncertainty and wide difference of opinion. Fundamental questions, such as the mechanism of immunity, the factors concerned in resistance and susceptibility, the part played by allergy and its relation to immunity, the relation of primary and secondary infection, await solution. Many types of animals have played an important part in the evolution of our knowledge of tuberculosis. A cooperative program of investigation on tuberculosis has been conducted at Northwestern University Medical School. On the assumption that the dog, because of its resistance and greater ability to localize the infection, is more like man than the experimental animals usually employed, it has been the chief animal used. Infection is induced by injecting a quantitated suspension of virulent tubercle bacilli in mucin deep into a selected bronchus by means of a special bronchoscope and catheter. The resulting local lesion in its development and progress is similar in many respects to pulmonary tuberculosis in man. Infected dogs react in a high percentage of cases to intracutaneous injection of Old Tuberculin or Purified Protein Derivative. They all show a significant increase in antibody titer as determined by the complement fixation test. In primary

infections the nature and development of X-ray shadows are similar to those seen in childhood tuberculosis.

THE IMPLICATIONS OF VARIABILITY ON THE SPECIES CONCEPT IN BACTERIOLOGY. *R. E. Buchanan*, Iowa Agricultural Experiment Station, Ames.

1. Among the bacteria each cell is to be regarded as an individual.

2. Bacteria respond readily to changes in environment; in many cases they also show marked genic instability.

3. Care must be exercised in distinguishing genetically expressed from environmentally impressed variations among bacteria.

4. There is no adequate proof either of sexuality or of its absence among the bacteria.

5. Evidence as to the presence or absence of a bacterial nucleus is still inconclusive, but that bacteria possess the functional equivalent of a nucleus is highly probable.

6. There is a tendency to confuse genic variations in the bacteria with some type of alternating mechanism corresponding to the alternation of generations in higher plants.

7. The generally accepted hypotheses used by geneticists to account for variation in higher forms, particularly the fungi, should be tested out as probably adequate for the bacteria.

8. Genic mutations may well account for most bacterial variation.

Equations are developed to illustrate what happens in a culture of bacteria when a mutation is being produced regularly in a definite fraction of the normal cells. When the assumption is made that the mutants increase at the same rate per cell as do normal cells it is shown that the number of generations needed to have half the cells in a culture mutants is inversely proportional to the proportion of normal cells which mutate in each generation. With a mutation rate of one in a million, it would require over a hundred years for one-half the cells in a culture to be mutants. If, however, the rate of increase per cell of the mutant is greater than the normal, that is, if the mutant is "better adapted," time is greatly decreased. For example, with a mutation rate of only one in a million, if the rate for the mutant is twice that for the normal, the mutants will equal the normal cells in number in less than a day in contrast to the hundred years when the increase rates are equal. The bearing of these facts and hypotheses upon nomenclature and classification of the bacteria is noted.

METHODS OF BACTERIOLOGICAL EXAMINATION OF CANNED FOODS. *G. V. Hallman*, Continental Can Co., Chicago.

In the examination of canned foods to determine the cause of spoilage, the presence of food-poisoning bacteria, the bacteria likely to produce spoilage, the sterility of canned foods or in inoculated packs to determine if the test organisms were destroyed, the preparation of the can, the methods for opening the can and the type of can opener used are all important in the prevention of contamination. Culture media, suitable for these bacteriological tests on canned foods are listed. For the examination of canned foods for sterility, the sample should be incubated and extreme care exercised to prevent contamination. Single colonies on culture plates should be viewed with suspicion until definitely proven to come from the product. When these occur, cans should be grossly overprocessed to insure their sterility and cultural methods checked

with these cans. The bacteriological findings with various types of canned food spoilage are listed.

THE INFLUENCE OF MINERAL FERTILIZERS ON SYMBIOTIC NITROGEN FIXATION. *J. L. Roberts and F. R. Olson*, Departments of Botany and Agronomy, Purdue University Agricultural Experiment Station, Lafayette, Indiana.

Greenhouse studies were made of the influence of phosphatic and potassic fertilizers on nitrogen fixation by Alsike clover and soybeans, growing in Plainfield sand and Clermont silt loam, and by alfalfa growing in Clermont silt loam. Phosphatic fertilizers greatly increased nitrogen fixation in all cases. Potassic fertilizer increased nitrogen fixation by alfalfa and by Alsike clover in Clermont silt loam. Potassium was apparently without effect on soybeans even when applied with phosphatic fertilizer. In Plainfield sand, Alsike clover responded to potassium only when phosphorus was also supplied.

More detailed studies with soybeans indicated that under our conditions a more or less constant amount of nitrogen was taken from the soil by the plant, regardless of the total nitrogen content of the plant. Hence, total nitrogen was an index of nitrogen fixation by the plants studied.

Nitrogen fixation by soybeans grown during winter months was not influenced by fertilizer treatment. Numbers and size of nodules on these plants were favorably influenced by fertilizer applications. Under these conditions environmental factors do not influence fixation and nodulation to the same degree.

PERCENTAGE UTILIZATION OF SUBSTRATES BY THE ROOT NODULE

BACTERIA. *R. H. Burris and P. W. Wilson*, University of Wisconsin.

Rhizobium meliloti (Wisconsin strain 131) grown on a mannitol medium proved unsuitable for studies of percentage substrate utilization because of the high endogenous respiration and the stimulation (about 150%) of this endogenous respiration when $M/10000$ 2:4 dinitrophenol (DNP) was added. Response of these organisms to DNP suggested that they differ from those whose substrate-utilizing ability has been reported by Clifton and coworkers. Addition of $M/10000$ DNP to suspensions of *R. meliloti* 131 supplied with glucose stimulated oxygen uptake whether added initially or after assimilation of the glucose was complete. The total oxygen uptake over a period of seven hours was the same with initial and delayed additions of DNP indicating that DNP was not acting upon an assimilatory mechanism.

Sugar analyses conducted simultaneously with oxygen uptake measurements showed a rapid disappearance of glucose. *Rhizobium trifolii* 205 gave 25.6% theoretical oxygen uptake before the glucose was exhausted in the absence of DNP and about 60% utilization in the presence of $M/10000$ DNP. The corresponding values for *R. meliloti* 131 were 8.6% and 13.4% oxidation. With *R. trifolii* 205 DNP exerted a marked sparing action on glucose, for in the presence of DNP glucose disappeared more slowly though oxygen uptake was stimulated 57%.

The data indicate that with gummy suspensions of rhizobia the stimulation in oxygen uptake induced by DNP additions can be attributed primarily to a stimulation of endogenous metabolism, whereas with less gummy suspensions the prevention of assimila-

tion is of greater quantitative importance.

TROUT FURUNCULOSIS—AN EPIZOÖTIC BACTEREMIA. *Winslow Whitney Smith*, Wisconsin Conservation Department, Madison, Wisconsin.

Trout furunculosis is a costly and widespread bacteremia which annually destroys millions of salmon and trout in hatcheries and natural waters throughout middle Europe, the British Isles, Canada, and the United States.

The disease resembles in certain respects the hemorrhagic septicemias of higher animals and it is caused by *Bacterium salmonicida*, an organism which has many characteristics of the *Pasteurella* group.

Trout furunculosis is a misnomer given the disease because of the bloody, serous lesions throughout the musculature which through the skin resemble boils. The disease attacks the intestine, liver, spleen, and kidneys, frequently liquefying the latter.

In the attempt to control this disease antibacterial antibodies have been induced in trout, carp, and turtles by injection of bacterin.

Another investigation has concerned the improvement of a method to disinfect the exterior of living trout eggs. A half-hour dip of trout eggs in 1:2000 acriflavine solution buffered at pH 7.8 is lethal for *B. salmonicida* and harmless to the trout eggs.

BACTERIOLOGY IN THE PAPER INDUSTRY. *Fred W. Tanner*, Department of Bacteriology, University of Illinois, Urbana, Illinois.

Bacteriology is playing an important role in many industries. One of the more recent ones to which it is applied for a definite purpose is the paper industry, especially that part which concerns manufacture of paper board

to be used for food containers. The sources of bacteria in paper are pulp, air in the mill, water supplies, fillers and sizes, contamination from unclean mills, and employees. Owing to its chemical nature, pulp may be an excellent medium for bacteria. The air in paper mills has also been found to contribute a few forms but this source is relatively unimportant. Water supplies may also furnish undesirable species if not rendered clean by filtration and chlorination. Paper and paper board usually have a low number of bacteria, averaging about 80 per gram. These are made up of harmless saprophytic species represented mainly by aerobic spore-forming organisms of no sanitary significance. This condition is easily explained by the fact that pulp is thoroughly cooked at high temperatures, chlorinated with heavy chlorine solutions and finally passed over drying rolls heated to high temperatures. The temperature of the drying rolls varies between 220-250°F. The roller revolves at a rate which exposes the paper at such temperatures from 1½ to 3 minutes. This is destructive to microorganisms. Coliform bacteria are not ordinarily present in high-quality paper board manufactured for the food industry. Pathogenic bacteria cannot survive the treatment just mentioned. Sanitary problems experienced in the paper industry are, therefore, not difficult in view of the treatment given pulp in its transformation into paper.

THE MORPHOLOGY AND PHYSIOLOGY OF CERTAIN MYXOBACTERIA OF IOWA. *J. M. Beebe*, Agricultural Experiment Station, Bacteriology Section, Iowa State College, Ames, Ia.

The morphology, colony characteristics and fruiting body formation of *Myxococcus fulvus, Myxococcus vires-*

cens, Polyangium fuscum and *Podangium erectum* are discussed as well as the distribution of the vegetative cells in the colony.

A relationship with other bacterial forms was noted and experiments showed that killed suspensions of various bacteria supplied all of the nutrient materials required for the growth of the myxobacteria under consideration.

Various carbon sources were utilized by the myxobacteria, including cellulose and starch; none of the carbohydrates were broken down sufficiently to give an acid reaction with bromcresol-purple.

FORMATION OF ACETYLMETHYLCARBINOL BY CELL-FREE JUICES. *M. Silverman and C. H. Werkman*, Bacteriology Section, Industrial Science Research Institute, Iowa State College, Ames.

The formation of acetylmethylcarbinol from pyruvic acid by an enzyme preparation extracted from *Aerobacter aerogenes* requires an acid reaction, phosphate, cocarboxylase, and manganese or magnesium. Acetaldehyde is not considered as a likely intermediate since it is not utilized by this enzyme system either in the presence or absence of pyruvic acid. The occurrence of a phosphorylated intermediate between pyruvic acid and acetylmethylcarbinol is indicated by a study of the influence of small additions of phosphate on the rate of CO_2 formation.

The overall reaction is expressed by the following equation:

$$2\,CH_3COCOOH \rightarrow 2\,CO_2$$
$$+$$
$$CH_3COCHOHCH_3$$
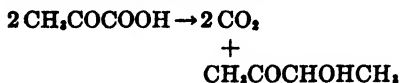
CELLULOSE DECOMPOSITION BY SOME AEROBIC ORGANISMS. *A. G. Norman and W. V. Bartholomew*, Iowa State College.

The decomposition of cellulose has usually been presumed to be purely hydrolytic in the primary stages. Glucose, enzymically produced, would then be the main fermentation substrate, and various workers have claimed to have demonstrated its production. Winogradsky made the suggestion that the action of many aerobic cellulose organisms is primarily oxidative, and stated that "oxycellulose" is a product of their action. Such an oxidative attack, if it proceeded uniformly, would preclude the possibility of glucose appearing as an intermediate. Oxidation might occur in two ways, affecting either the terminal carbonyl group of the chain or the projecting carbinol groups on carbon atom 6 in each glucose unit. The former would result in the production of carboxyl groups of the gluconic type, and the latter of groups of the glucuronic type. Products of a polyuronide nature have been obtained from cellulose fermentations with several active aerobic organisms and the presence of uronic groups detected in the unfermented residue of the fibres.

THERMOPHILIC FLAT-SOUR ORGANISMS IN SOME MATERIALS USED IN FOODS. *F. M. Clark*, Department of Bacteriology, University of Illinois.

Thermophilic bacteria have undoubtedly been the cause of a great amount of spoilage in canned foods. Since early work had shown that the spores of these organisms were not numerous on the raw product, search was made for the source of the organisms. Probably the first material added to food which was incriminated was sugar. It was shown that the presence of spores of thermophilic organisms in sugar could seed various parts of the factory and thus cause a spoilage of the canned product. Since

methods of analysis and standards of quality have been set up, the bacteriological quality of sugar has improved greatly. Starch has also been found to carry thermophilic spoilage organisms into the canning factory. A comparison between these flat-sour spoilage organisms isolated from starch and those isolated from sugar indicates that they may not be exactly alike. Those from sugar seem to be mainly obligate thermophiles probably identical with *Bacillus stearothermophilus* Donk. From starch the thermophilic flat-sour spoilage organisms are mainly facultative thermophiles. Recent work on gelatin indicates that it also contains thermophilic flat-sour organisms similar to those isolated from starch and sugar.

NON-SPORE-FORMING THERMODURIC BACTERIA IN MILK. *H. Wainess and E. H. Parfitt*, Dairy Department, Purdue University, Lafayette, Indiana.

A new thermoduric organism was described and named "*Sarcina thermodurica*," based on its ability to resist the heat treatment of both the holding and the short-time-high-temperature methods of pasteurization. Agar colonies were small, yellow, convex, entire and glistening and a slight acidity was produced in litmus milk after three to four days incubation. The organism can be isolated from milking machines and other dairy farm utensils.

It was also found that no relation existed between the organism's physiological age and its resistance to pasteurzation temperatures.

The methylene blue test, in cases where the milk is to be pasteurized, affords an unreliable index of the final thermoduric population and of the conditions under which the milk has been produced.

A method of detecting the presence of thermoduric organisms in the raw milk supply, called a "Pasteurization Test," was described.

HEAT-RESISTING BACTERIA IN MILK. *M. J. Prucha and A. C. Maack*, Department of Dairy Husbandry, University of Illinois, Urbana, Illinois.

Heat-resisting bacteria, sometimes called thermoduric, may be described as non-spore-bearing bacteria which are not destroyed by pasteurization at 143°F. for 30 minutes. They are not thermophiles because they do not multiply at this temperature. For the most part they are gram-positive, coccus forms. One of such strains of the organism required 4 hours exposure to 143°F. before all the cells were destroyed.

This group of organisms is also resistant to chlorine disinfectants. One of the organisms which caused high bacterial counts in pasteurized milk survived one minute exposure to a disinfecting solution containing 100 ppm. of active chlorine.

Their principal habitat appears to be the utensils especially when these are not kept scrupulously clean.

For some unknown reason this group of bacteria appears to be troublesome especially during January, February, March, and April.

GAS PRODUCTION IN NITRATE SUGAR MEAT MEDIA BY THE GENUS BACILLUS. *L. B. Jensen and W. R. Hess*, Swift & Company, Research Laboratories, Chicago, Illinois.

Certain species of the genus *Bacillus* are of great economic importance in the meat industry, owing to their marked powers to ferment sugars with the production of gas in the presence of nitrate and cured meat. In addition,

a red chromogenic species often discolors the dried Xarque or jerked beef of South America. We also call attention to the presence of spice contamination and food-wrapping-paper contamination with the spores of this genus. A medium is described to detect these gas-forming bacteria. In standard sugar broths, no gaseous fermentation takes place, but in nitrate-sugar-pork media these bacilli form voluminous quantities of CO_2 gas. Vitamin B_1, or thiamin chloride, induces gas production with a few strains in ordinary sugar-nitrate broths without addition of cured meat.

THE RELATIONSHIP OF CO_2 UTILIZATION TO SUCCINIC ACID FORMATION BY THE COLIFORM BACTERIA. *H. G. Wood and C. H. Werkman*, Bacteriology Section, Iowa Agricultural Experiment Station, Ames.

The inhibition of fixation of CO_2 by NaF in the propionic acid fermentation has been shown (Biochem. J., **34:** 129-38, 1940) to cause a mole-equivalent decrease in the formation of succinic acid. This is interpreted as additional evidence that succinic acid may be formed by union of 1- and 3-carbon compounds.

The formation of succinic acid from different substrates by coliform bacteria is not uniformly inhibited by NaF. The yield of succinic acid from glycerol and citrate is not influenced by the presence of fluoride. The implication is that succinic acid may be formed without utilization of CO_2 in these fermentations. In the case of the fermentation of galactose and pyruvic acid, succinic formation is inhibited by fluoride. It is probable that there is utilization of CO_2 in these fermentations.

THE STIMULATIVE EFFECT OF GLUCOSE ON THE ANAEROBIC DISSIMILATION OF CITRATE BY STREPTOCOCCUS PARACITROVORUS. *Hutton D. Slade and C. H. Werkman*, Bacteriology Section, Iowa Agricultural Experiment Station, Ames.

Streptococcus paracitrovorus is unable to utilize citric acid as a sole source of carbon; however, in the presence of glucose, the acid is dissimilated. This reaction has been studied manometrically and by the use of resting cell fermentations. The stimulative effect of glucose in this system is very marked. Under these conditions, a glucose concentration of at least one-twentieth that of citrate is necessary for the complete dissimilation of the acid. Galactose, lactose, fructose and mannose can replace glucose.

The reaction is poisoned by sodium fluoride, and monoiodoacetate. Sodium azide stimulates the anaerobic dissimilation of glucose in the presence of citrate, thereby providing sufficient activation for citrate fermentation.

By use of resting cell suspensions, a complete dissimilation of citric acid has been obtained during an incubation period of 4-5 days. The final products have been identified as CO_2, H_2, formate, acetate, lactate and succinate.

It has been impossible to show that stimulative effect of glucose is due to donation or acceptance of hydrogen. It thus appears that glucose is serving in the synthesis of some enzyme or respiratory carrier which enables citrate dissimilation to proceed normally. No evidence to this effect has been obtained.

THE RESPIRATION OF LACTOBACILLUS BULGARICUS NEAR ITS MAXIMUM GROWTH TEMPERATURE. *Robert M. Stern and W. C. Frazier*, Department of Agricultural Bacteriology, University of Wisconsin.

Investigations of the oxygen uptake of "resting" cells and of growing cells

were carried out at 37°C. and at 49.5°C. by means of the Warburg respirometer; and amounts of growth were estimated by use of the Evelyn photoelectric colorimeter.

Results with "resting" cells indicate that during the first 30 minutes of the respiration there is a more rapid rate of oxygen uptake at 49.5°C. than at 37°C., but that at the higher temperature the rate drops off rapidly and the total oxygen uptake after ninety minutes is greater at 37°C. than at 49.5°C., indicating a marked degree of inactivation of the respiratory mechnism at the higher temperature. Incubation of the "resting" cells at the two temperatures for periods of 1, 2, and 4 hours, respectively, followed by a determination of oxygen uptake at 37°C., suggests a greater inactivation at 49.5°C. than at 37°C., especially with those cells incubated for 4 hours.

Investigations of the respiratory activity of growing cells indicate that during the first few hours both the rate of growth and of oxygen uptake are greater at 49.5°C. than at 37°C. At the higher temperature the maximum amount of oxygen uptake and of growth is obtained between the 4th and 5th hours; then the oxygen uptake decreases rapidly, practically ceasing by the 7th hour. Activity at 37°C., however, continues at an increasing rate until the 7th or 8th hour; then gradually decreases and the cells become relatively inactive by the 11th or 12th hour.

The results on both "resting" cells and growing cells suggest that the cessation of growth at the high temperature probably is due to the inactivation of the respiratory mechanism.

BACTERIOLOGY OF BRICK CHEESE. *E. M. Foster, J. C. Garey, and W. C. Frazier*, Department of Agricultural Bacteriology, University of Wisconsin.

Studies on the bacteriology of Brick cheese show that in cheese made with *Streptococcus lactis* alone the number of lactics increased rapidly to one to four billions per gram within 12–24 hours. By this time the lactose usually had disappeared and the pH was about 4.75–4.90. When *Streptococcus thermophilus* was used alone, growth was most rapid during and just after cooking, but slowed down greatly when the curd cooled to room temperature. The pH reached 5.20–5.40 and considerable lactose remained in the curd. Later, however, *Streptococcus lactis* organisms from the milk multiplied and lowered the pH further. Gas was produced in this cheese by a spore-forming anaerobe prior to the secondary drop in pH.

Use of the washed curd process made it possible to regulate the point to which the pH would drop when all the residual lactose was fermented. The most desirable acid formation resulted from a combination of 0.5 per cent *Streptococcus lactis* and 0.5 per cent *Streptococcus thermophilus*.

Streptococcus lactis was eventually the predominant organism in all cheese made. *Streptococcus thermophilus* decreased rapidly in numbers even when it was the starter organism. A few species of lactobacilli, notably *Lactobacillus casei*, developed in the raw milk cheese, and occasionally in pasteurized milk cheese, after about 2–3 weeks. They gradually increased in numbers thereafter.

SOME MILK PLANT CONTROL PROBLEMS OF A BACTERIAL NATURE. *P. H. Tracy*, Department of Dairy Husbandry, University of Illinois, Urbana, Illinois.

Refinement of the methods of production and distribution of milk has

been such that today there can be no question regarding the safety of that portion of our milk supply that is under competent supervision. In 1938 in the entire United States there were only 1244 cases of illness and 21 deaths traceable to milk and other dairy products, and in none of these cases were pasteurized milk or milk products involved.

Although there has been progress made in improving the sanitary quality of our milk supplies certain problems have evolved from the new order of things. Health regulations governing the production and marketing of milk and dairy products have been developed more or less in accordance with the ideas of local officials. The final objective has been the same in all cases—safety—but the methods have varied. There also exists a lack of co-operation between the inspection services of most of the different markets.

This results in what is essentially a trade barrier based on health regulations. Economic aspects of public health regulations are beginning to be of paramount importance. The higher standards placed on milk to be used for fluid milk purposes than those placed on milk to be used in the manu-facture of evaporated milk makes the situation more favorable for a lower market price for canned milk. Differences in standards on two adjacent markets result in unfair competition between dealers in those markets.

There are certain quality problems that have resulted either directly or indirectly from the general elevation of the quality of milk. Oxidized flavor is more apt to occur in milk of low bacterial content. Many housewives complain today that their milk no longer sours normally, but develops putrid flavors and odors upon storage. Due to its better keeping quality, milk is sometimes abused by permitting it to stand in windows or on porches exposed to the sunlight resulting in a noticeable flavor defect due to the action of the sun's rays on milk protein. Thermoduric and thermophilic bacterial defects seem to be more of a problem today than formerly.

We should not emphasize sanitary measures beyond the point where practicability ceases. What we need is not only a safe milk supply but one of pleasing flavor and of such a price that families with limited incomes will feel that they can afford to buy milk regularly and in quantities commensurate with their needs.

OHIO AND MICHIGAN BRANCHES

TOLEDO, OHIO, MAY 4, 1940

OBSERVATIONS ON THE DEVELOPMENT OF RESISTANCE TO SULFAPYRIDINE BY DIPLOCOCCUS PNEUMONIAE. *H. A. Dettwiler and L. H. Schmidt*, Christ Hospital Research Institute and Department of Biochemistry, College of Medicine, University of Cincinnati, Cincinnati, Ohio.

Strains of Types I and III pneumococcus, carried serially through groups of mice treated with sulfapyridine, developed increased resistance to the drug. After 5 to 9 successive transfers, resistance was so well developed that the survival time of treated mice approached that of untreated controls. With one of the Type III strains, this property of sulfapyridine resistance was not altered appreciably by 12 serial passages through untreated

mice, was diminished slightly after 26 such passages, and was lost entirely after 53 passages.

A comparison of the *in vitro* growth of this Type III resistant strain with the corresponding parent organism showed that 15 mg. per cent sulfapyridine did not restrain the growth of the resistant organism but did inhibit the growth of the parent strain.

A strain of Type II, normally unable to grow in 15 mg. per cent sulfapyridine *in vitro*, was carried through a series of experiments in which the concentrations of the drug were successively increased. In this manner this strain was trained to grow well in a blood-broth medium saturated with sulfapyridine. The saturated solution contains 120 mg. per cent at 37°C.

The resistant strains, whether obtained by *in vivo* or *in vitro* methods, showed no alteration when typed by the Neufeld reaction. Changes in morphology and virulence were absent.

THERAPEUTIC SUFFICIENCY TESTS IN PNEUMOCOCCIC AND STREPTOCOCCIC INFECTIONS. *Bernard Steinberg and Ruth Martin*, City Hospital, Toledo, Ohio.

Treatments of pneumococcic and streptococcic infections are necessarily haphazard since no quantitative relationship between the therapeutic material and the degree of infection is available. Clinical manifestations constitute the only present criteria for adequate or insufficient treatment, Experimental evidence is presented, using peritoneal infections by the pneumococcus and the streptococcus, that the morphologic appearance of these microorganisms and their relation to the phagocytic cells offer early criteria for a quantitative determination of treatment.

ACIDURIC BACILLI AND DENTAL CARIES. *Marshall L. Snyder*, University of Michigan, Hygienic Laboratory, Ann Arbor, Michigan.

It was shown that, of the many bacterial types in the mouth, only lactobacilli and yeasts could be correlated with caries activity on the basis of incidence and numbers. Using the numbers of lactobacilli, quantitatively estimated, as an index, it was possible to diagnose caries in ninety per cent of cases. Yeasts occurred more frequently in carious than in caries-free individuals, but the frequency of isolation was small compared with that of lactobacilli.

A simple colorimetric method was described for the diagnosis of caries activity. The results obtained with this test approached the condition established by clinical examination for 63 young children more closely than the diagnosis made by lactobacilli counts. Since the test reflects acid production by any of the organisms inoculated into the selective medium (brom-cresol-green glucose agar—pH 4.7–5.0), it is apparent that no aciduric or acidogenic bacteria can be neglected. However, lactobacilli are the bacteria primarily responsible for the color changes induced by acid formation. The rate of action is apparently accelerated by yeasts which play only a secondary rôle in caries activity.

A DISCUSSION OF THE MICROBIAL FLORA OF INSECTS. *Edward A. Steinhaus*, Department of Bacteriology, Ohio State University, Columbus, Ohio.

The microbial flora of insects may be divided into two large groups: extracellular and intracellular. The extracellular flora is concerned chiefly with the bacteria, yeasts, and molds which

occur freely in the alimentary tract of the insect. By the term "intracellular flora" we mean those microbes living within the very tissue cells of the host.

In a survey of the extracellular bacterial flora of certain insects, most of the major types of bacteria were found. Eighty-three strains of bacteria, 2 of yeasts, and 2 of molds were isolated from 28 species of insects. The insects examined included species from 7 orders of the class Hexapoda. Of the bacteria isolated, 45 (54%) were gram-negative short rods, 14 (16%) were gram-positive cocci, 12 (14%) were gram-positive spore-formers. Based on cultural and physiologic characteristics, 31 new species were represented among the 83 strains. Twenty (44%) strains of the gram-negative short rods were coliforms.

The bacterial flora may differ in the various parts of the alimentary tract. For example, the milkweed bug (*Oncopeltus fasciatus*) had a flora in its pylorum and rectum distinctly different from that in the four stomachs which precede them.

THE PRESENCE OF A CAPSULE ON BRUCELLA CELLS. *I. Forest Huddleson*, Department of Bacteriology and Hygiene, Michigan State College, East Lansing, Michigan.

All smooth strains of *Brucella* have been found to possess a definite capsule. Dissociated forms do not show a capsule. The technic used for demonstrating the capsule is a modification of the Burri India ink method.

The size of the bacterial cells plus capsules varies from 1.5 to 2.5 micra. Capsular swelling has not been demonstrated in the presence of antiserums.

THE CULTURE METHOD FOR THE DETECTION AND ISOLATION OF NEISSERIA GONORRHEAE. IMPORTANCE AND METHODS OF SUPPLYING CARBON DIOXIDE. *C. W. Christensen and H. W. Schoenlein*, Difco Laboratories, Incorporated, Detroit, Michigan.

Four-hundred-and-eighteen specimens from chronic and treated cases of gonorrhea in females, cultured on Bacto-Proteose No. 3 Agar enriched with whole blood or Bacto-Hemoglobin, yielded 76 positives for the gonococcus, as compared with 17 positives obtained by microscopic examination of the exudates. One specimen was positive by microscopic examination which was missed by culture.

Specimens incubated under a partial carbon dioxide tension yielded a higher percentage positive for *Neisseria gonorrheae* than did those incubated in moist containers without added carbon dioxide.

For the primary isolation and cultivation of gonococci, three simple reliable methods of supplying a satisfactory CO_2 tension were studied. Each of these three gave results equal to those obtained by supplying ten per cent CO_2 from a tank. These three methods are as follows:

1. Place a lighted smokeless candle inside and near the top of the container in which the cultures are incubated and place the cover on tightly;

2. Place moistened oats in the bottom of the container in which the plates are incubated and put the cover on tightly; (A 200 ml. beaker of oats is adequate for a container of 2½ liters capacity.)

3. Place a small flask containing dilute sulfuric acid in the container with the plates and add sodium bi-

carbonate before placing the cover on tightly. (One gram Na HCO₃ per 3 ml. concentrated H₂SO₄, diluted to 100 ml., is adequate for a container of 2½ liters.)

REMARKS ON THE SYRIAN HAMSTER, *J. A. Doull*, Western Reserve University, Cleveland, Ohio.

PULLORUM DISEASE CONTROL. *E. W. Roberts*, Animal Disease Laboratory, Reynoldsburg, Ohio.

RESEARCH ON THE RICKETTSIAE OF TYPHUS IN MEXICO CITY, *N. Paul Hudson*, Department of Bacteriology, Ohio State University, Columbus, Ohio.

EASTERN NEW YORK BRANCH

RENSSELAER POLYTECHNIC INSTITUTE, TROY, N. Y., APRIL 26, 1940

PORPHYRIN PRODUCTION BY CORYNEBACTERIA. *Mary W. Wheeler*, Division of Laboratories and Research, New York State Department of Health, Albany.

Studies of porphyrin synthesis have been extended to include, besides virulent, toxigenic diphtheria bacilli, both nonvirulent strains of this microorganism and strains of related species or corynebacteria.

The presence of porphyrins was assumed if the glacial acetic acid plus ether extract of the culture filtrate was straw colored or salmon pink to coppery red. A number of these extracts were also examined spectroscopically by M. O'L. Crowe. In each instance fluorescence and absorption bands were observed in positions characteristic of the porphyrins.

Under the conditions of the experiments, porphyrins were observed in cultures of ten nonvirulent, nontoxigenic strains of *Corynebacterium diphtheriae*, of three strains of *C. ulcerans*, two of *C. ovis*, and one of *C. hoagii*, but not in cultures of three strains of *C. xerosis* and one of *C. hofmanni*.

Further analysis of the cultures suggested that the conditions under which the nonvirulent diphtheria bacilli and related species of coryne-

bacteria synthesize porphyrins are similar to those under which pigment and toxin production are observed in cultures of virulent, toxigenic diphtheria bacilli.

THE FIVE STRUCTURES OF BACTERIAL COLONIES. *R. V. Hoffman* (by invitation).

IN VITRO STUDIES ON THIAZOL DERIVATIVES OF SULFANILAMIDE. *C. A. Lawrence*, Research Laboratories, Winthrop Chemical Company, Inc., Rensselaer, N. Y.

Sulfathiazol, sulfamethylthiazol and sulfaphenylthiazol were found to be superior to sulfapyridine and sulfanilamide in their bacteriostatic effects upon dilute suspensions of *beta* hemolytic streptococci (C203), pneumococci Types I, II and III and *Staphylococcus aureus*. The extent of growth in the test solutions after 24–26 hours incubation at 37°C. was determined by pouring plates and counting the developing colonies.

These results confirm earlier findings wherein, under different experimental conditions and using large inocula, the thiazols proved to be more active than sulfanilamide and sulfapyridine upon the organisms mentioned.

The bacteriostatic actions of sulfathiazol and sulfamethylthiazol were found to be somewhat greater than those of sulfapyridine, sulfaphenylthiazol and sulfanilamide upon several strains of *Eberthella typhosa* and *Salmonella* organisms, two strains each of *Escherichia coli* and *Aerobacter aerogenes*, and one strain each of *Shigella dysenteriae* and *Proteus vulgaris*. The observed effectiveness of the compounds from high to low was in the order named.

OBSERVATIONS ON PNEUMOCOCCUS CARRIERS IN A RURAL COMMUNITY. *G. M. Mackenzie, M. D., Jay Tepperman, M.D., and Thistle M. McKee, M.D.*

A study of carriers in a village of 250 people, in which 5 cases of Type I pneumonia had occurred within 8 weeks, indicated:

(1) That the observed carrier rate was significantly influenced (a) by the frequency of repetition of the cultures and (b) by the methods used to isolate pneumococci from carriers. (2) For the 14 months, during which 7 surveys were made, the Type I carrier rate was 24.3 per cent; for pneumococci of all types, including Type I, it was 73.4 per cent; the multiple type carrier rate was 41.3 per cent. (3) Five months after the occurrence of the last case of pneumonia the carrier rate was 5.9 per cent. (4) After Type I, the types of pneumococci most frequently isolated were Types VI, III, XIII. Eighteen per cent of the carriers harbored pneumococci, which did not react with the specific sera of Types I-XXXII. (5) The chief determinant of the Type I pneumococcus carrier state seemed to be the amount of contact with carriers. No definitely positive correlation was demonstrated between the carrier state and age, season, overcrowding or upper respiratory infections.

BROTH FOR THE PRODUCTION OF PNEUMOCOCCUS SOLUBLE SPECIFIC SUBSTANCES. *Lucena K. Robinson,* Division of Laboratories and Research, New York State Department of Health, Albany.

A beet-sugar peptone water prepared from dialyzed Nutri-Peptone has been found superior to media prepared from other peptones tried for the growth of pneumococci for the isolation of soluble specific substances. In this medium young culture filtrates relatively rich in soluble specific substance are obtained. The addition at the time of inoculation of a phosphate carbonate buffer solution to the previously sterilized medium gives a further increase in the yield of soluble specific substance. Broth constituents and bacterial proteins are readily eliminated in the subsequent purification procedures.

Nutri-Peptone has a high ash, the iron content of which is relatively great. However, removal of all but a trace of iron from the medium by calcium phosphate precipitation did not affect significantly the growth of pneumococci. Nutri-Peptone is markedly rich in nitrogenous constituents that dialyze through the cellophane membrane. The Nutri dialyzate contains 45 per cent of the original peptone nitrogen as against 29 per cent in dialyzates from Difco proteose and Parke Davis peptones.

TITRATION OF ANTIPNEUMOCOCCUS RABBIT SERA BY COMPLEMENT FIXATION. *Christine E. Rice,* Division of Laboratories and Research, New York State Department of Health, Albany.

The complement-fixing activity of antipneumococcus rabbit sera (types

I–XXXII) with chemically purified homologous type-specific polysaccharide as antigen has been studied by the quantitative technic.[1] The relative amounts of serum and antigen used and the time and temperature of fixation had a marked effect on the reaction obtained.

These systems were particularly sensitive to the presence of an excess of antigen. When adjusted to give the maximum reaction, conventional linear relationships between serum and antigen obtained over the complement range studied. Considerably more complement was fixed during a fixation period of twenty-four hours at 3–6°C. than during one and one-half hours at 37°C.; proportionately more antigen was required for maximum fixation at the lower temperature. Linear relationships between complement and serum in the presence of the maximally reactive dose of antigen were usually observed at both temperatures; certain antisera, notably for type I, were exceptional in this respect.

These results are being considered in relation to those of other methods of determining activities of the same sera.

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[1] Wadsworth, A. B., Standard Methods of the Division of Laboratories and Research of the New York State Department of Health, 2d ed., Baltimore, The Williams & Wilkins Company, 1939, pp. 213–267.

ENDOCARDITIS IN RABBITS: PRELIMINARY REPORT. *John K. Miller*, Division of Laboratories and Research, New York State Department of Health, Albany.

The hearts of 800 rabbits were examined. Twenty rabbits had been immunized with intravenous injections of living type-III pneumococci; all others received killed pneumococcus vaccine of a number of types. Cardiocentesis was performed on all but 67 rabbits. Thirty per cent of the hearts had thickened, opaque mitral cusps; these were as frequent in rabbits without cardiopuncture as in those repeatedly bled from the heart. Endocarditis was found in 24 hearts, the aortic valve being involved 3 times and the mitral valve 21 times. Two immunized rabbits with endocarditis had not been bled from the heart. With but two exceptions, the sera were of high potency.

Microorganisms resembling *Pasteurella lepiseptica* were isolated from 3 mitral valves and 1 associated blood culture. A pure culture of an, as yet unidentified, gram-negative, rod-shaped bacterium was recovered from the blood and aortic valve of 1 rabbit. On microscopic examination gram-negative, rod-shaped microorganisms were seen in the valvular tissues of 3 other rabbits. Type-III pneumococci were isolated from the blood and aortic valvular lesion of 1 rabbit.

CENTRAL NEW YORK BRANCH

CORNELL UNIVERSITY, ITHACA, N. Y., MAY 18, 1940

THE SELECTIVE DISINFECTION OF SEEDS. *Herbert S. Hamilton and Alvin W. Hofer*, New York State Agricultural Experiment Station, Geneva, New York.

In routine testing of commercial legume-inoculants, it is often desirable to free seeds from nitrogen-fixing bacteria. For certain research purposes, it would also be helpful to

destroy all microorganisms carried by seeds. An investigation of seed-borne bacteria and fungi has shown that these are carried in the interiors of the seeds as well as on the surfaces. The problem of destroying these organisms is complicated by the fact that the viability of the seeds is easily injured. The difficulty is increased further by the fact that practically all seeds of the common plants so far studied have carried these microorganisms.

A solution of the problem has been attempted through the use of chemical disinfection. Disinfectants with established properties have been used in known concentrations for the immersion of whole seeds, chiefly those of legumes. No technique or disinfectant was found effective in destroying the bacteria within seeds, without at the same time materially reducing their ability to germinate. The surface, on the other hand, has been relatively easy to sterilize without injuring the seeds unduly, thus obtaining a partial or "selective" disinfection.

A technique has been developed to study the resistance of bacteria within seeds. It has been found that failure to disinfect the interior of the seeds is due to physical and chemical factors rather than to a natural resistance of the microorganisms.

⊘THE DEMONSTRATION OF PHOSPHATASE IN CERTAIN THERMOPHILIC SPECIES OF THE GENUS BACILLUS. *Harold W. Leahy,* Rochester Health Bureau Laboratories, Department of Bacteriology, School of Medicine and Dentistry, University of Rochester, Rochester, New York.

In continuation of previous studies, phosphatase has been demonstrated in four species of the genus *Bacillus; Bacillus non-diastaticus,* Prickett BG-9, *Bacillus kaustophilus,* Prickett BD-53, *Bacillus stearothermophilus,* Shaw I-5, and an unnamed but well described thermophile, *Bacillus sp.,* Cameron and Esty No. 1356.

The optimal hydrogen-ion concentration of the lyophilized cells was determined by the method described previously (J. Bact. *38,* 117, 1939), except that the organisms were grown on tryptone-glucose extract milk agar at 50°C. Cultures of the organisms grown in skim milk at 50°C. were also examined by the Kay-Graham and modified Scharer tests.

The lyophilized preparations from all four strains exhibited a weak phosphatase activity. The optimal hydrogen-ion concentration, however, varied with each strain, but fell within the range of from pH 5.0 to pH 6.2. On the other hand, none of the four species, when grown in skim milk, interfered with either the Kay-Graham or the modified Scharer tests. The non-interference of the bacterial phosphatase with these tests, as applied to milk, is explained upon the basis that the optimal conditions for the activity of bacterial phosphatase (pH 5.0–6.2) do not coincide with those of the tests (pH 8.9).

THE ADAPTIVE ENZYMES OF CERTAIN STRAINS OF YEASTS. *Harry Rhoades,* N. Y. State Agricultural Experiment Station, Geneva.

THE PENETRATION OF RADIOACTIVE PHOSPHORUS INTO ENCYSTED TRICHINELLA LARVAE. *O. R. McCoy, V. F. Downing and S. N. Van Voorhis,* University of Rochester, Rochester.

GASTROINTESTINAL WORM PARASITISM ACQUIRED BY SHEEP DURING THE WINTER SEASON. *Donald W. Baker and John Sawyer,* Parasitological

Laboratory, New York State Veterinary College.

Observations made over a period of five years suggested that sheep and goats, while stabled during the winter months, were parasitized by nematodes of the various strongyloid types, as well as by tapeworms. The epizoological significance of these observations was established by the controlled studies of Scrivner and Baker which were made during the winter of 1938–1939. The present report verifies and extends their findings.

Lambs known to be free from intestinal parasites were transferred to a new environment in which the bedding was contaminated by the feces of adult sheep. Experiments were conducted during cold winter weather when the only possible source of infection was from the infested indoor environment. The lambs fall into 2 groups. In group A were 5 lambs, 8 or 9 months old, that had been reared parasite-free. One of these was kept in the laboratory as a control. Group B consisted of 13 lambs, born during December, January and February and transferred to the infested environment immediately after birth. Fecal examinations were made at weekly intervals. Both groups acquired various species of the superfamilies *Strongyloidea* and *Trichuroidea*, and tapeworms of the genus *Moniezia*. The first positive fecal examinations were obtained 54 days after birth for Group B, and 42 days after exposure for Group A. The recorded exposure period for Group B would appear to be from 14 to 21 days too long, since lambs eat little formed food during early life. These experiments establish that gastrointestinal worm parasites can be acquired by sheep while stabled during the winter months.

A MOTOR-DRIVEN, MECHANICALLY SUPPORTED ULTRACENTRIFUGE FOR THE SEPARATION OF BIOLOGICAL MATERIALS. *Arthur J. Rawson, Henry W. Scherp and F. E. Lindquist*, Eldridge Reeves Johnson Foundation for Medical Physics and Departments of Pediatrics and Bacteriology, School of Medicine, University of Pennsylvania, Philadelphia, Pa., the Children's Hospital of Philadelphia, and Department of Bacteriology, School of Medicine and Dentistry, University of Rochester, Rochester, N. Y.

A motor-driven, mechanically supported ultracentrifuge has been devised for the separation of biological materials. This centrifuge is similar in principle to that of Bauer and Pickels: a conical rotor carrying containers filled with the material under investigation is suspended and spun inside an evacuated chamber by means of a flexible shaft. The supporting and driving mechanism, however, is a "lathe-grinder." The use of a motor-drive and mechanical support affords advantages in respect to cost, simplicity of operation, and ease of maintenance.

A maximal speed of 44,000 r.p.m. has been attained, giving a maximal "tube-field" of 150,000 times gravity in the rotor employed. The centrifuge has been extensively used for the separation of influenza virus and has been shown to be capable of sedimenting proteins of molecular weight as low as 309,000, *i.e.*, edestin, within a period of several hours.

THE PROTECTIVE VALUE OF IMMUNE SERA AGAINST MURINE INFECTION PRODUCED BY HEMOPHILUS PERTUSSIS. *W. S. Bradford and H. W. Scherp*, University of Rochester, Rochester.

A COMPARISON OF DIFFERENT AEROBES UNDER ANAEROBIC CONDITIONS. *G. L. Richardson*, College of Agriculture, Cornell University, Ithaca.

A WIDE-RANGE H-ION INDICATOR PRODUCED BY AN ACTINOMYCES SPECIES. *Jean E. Conn and H. J. Conn.*

During the course of an attempt to explain the color variation of the pigment that was produced by a single species of *Actinomyces* under different conditions, one culture was observed to change in color from red to coral when the final H-ion concentration of the medium was increased. By growing the organisms in quantity and extracting them with alcohol, pigment in alcoholic solution was made available for further study. It was found that the color of the pigment in alcoholic extract changed from orange to yellow on addition of H_2SO_4, and to violet on addition of NaOH. When the alcoholic extract of the pigment was added to buffered solutions of known pH, it acted as a H-ion indicator. The color changed from yellow at pH 5, through red at neutrality, to violet at pH 8.5.

It is suggested that the pigment may serve bacteriologists as a wide-range indicator, if it is found to indicate pH values accurately, and if it can be produced in quantity. No attempt has been made to identify the pigment chemically.

A SYNTHETIC MEDIUM FOR STREPTOCOCCUS RHEUMATICUS. *R. L. Schuman and M. A. Farrell*, Pennsylvania State College, State College, Pennsylvania.

Streptococcus rheumaticus, a non-hemolytic pathogenic organism, has been found to grow upon a completely synthetic medium. Pantothenic acid, Vitamin B_6 and riboflavin in optimal concentrations of 1.5, 0.5 and 0.3/ml.,

respectively, were essential. The basal medium consisted of glucose, a salt mixture and seven essential amino acids, tryptophane, valine, methionine, tyrosine, lysine, arginine and glutamic acid. Beta-alanine could not replace pantothenic acid in the synthetic medium devised. The growth of *Streptococcus rheumaticus* in this medium was found to be quantitatively equal to that yielded by a complex, nonsynthetic medium containing principally a hydrolysate of gelatin and a purified concentrate of liver extract. Other investigators have reported substances that stimulate the growth of certain hemolytic streptococci. These substances, glutamine, glutathione, betaine, nicotinic acid, glucosamine, uracil, xanthine and hypoxanthine, did not measurably increase the growth of *Streptococcus rheumaticus*. The presence of reducing materials, such as sodium sulfide and reduced iron, was found to have no noticeable effect.

Experiments are now in progress to determine the applicability of this synthetic medium to the growth of other strains of streptococci.

SEED-BED "STERILIZATION" AND TOBACCO WILDFIRE. *J. Naghski, D. E. Haley and J. J. Reid*, Pennsylvania Agricultural Experiment Station.

The steaming of seed-beds, originally introduced as a measure for the control of weeds, has been advocated for the control of tobacco wildfire. Observations in Pennsylvania and elsewhere, however, have given the impression that wildfire is more prevalent after seed-beds have been steamed in the spring than when the beds were not steamed, or were steamed the previous fall.

We have studied this problem. Samples of soil were taken at seeding-

time from beds that were steamed in the fall, in the spring, or not steamed, respectively. Glucose-peptone-acid agar was used to count the fungi present; nutrose agar, the bacteria and actinomyces; and crystal violet-asparagine-mineral salts broth, the green-fluorescent bacteria.

All samples yielded fungi, bacteria and actinomyces. Green, fluorescent, gram-negative rods were present in every sample, but were most numerous in soils steamed in the spring. Serological tests established that 75 per cent of the green-fluorescent organisms from steamed soils and 60 per cent from soils not steamed were identical with known cultures of the wildfire organisms.

Recommendations, therefore, that steaming serves as an effective measure for eliminating wildfire organisms from seedbeds are misleading.

THE SIGNIFICANCE OF THE GAS RATIO IN DISTINGUISHING MEMBERS OF THE PROTEUS AND RELATED GROUPS. *M. L. Speck and C. N. Stark*, Department of Dairy Industry, Cornell University, Ithaca.

VIRULENCE OF SALMONELLA TYPHIMURIUM

I. Analysis of Experimental Infection in Mice with Strains of High and Low Virulence

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In comparing the antigenic structure, the virulence and the immunizing capacity of smooth and rough strains of *Salmonella typhimurium* (Mackenzie, Fitzgerald and Pike, 1935) we observed that a large decrease in virulence may occur without demonstrable changes in other biological characters, and that the smooth somatic antigen may be qualitatively and quantitatively the same in strains of high virulence and strains of low virulence. With regard to active immunization our findings confirmed the observations of others—immunization is accomplished only with strains containing the smooth somatic antigen. Our failure to identify with virulence any of the common biological characters of smooth strains led to the experiments reported in this paper; they represent (1) a more complete study of the virulence of the strains of *Salmonella typhimurium* used and (2) an attempt to evaluate as possible determinants of virulence certain other biological characters, namely, invasiveness, resistance to phagocytosis, ability to survive, and ability to multiply in the host.

MATERIALS AND METHODS

Four strains of *Salmonella typhimurium* have been employed. Their biological characters, including immunizing properties and virulence, have been described elsewhere (Mackenzie, Fitzgerald and Pike, 1935). Since the time of that publication we have observed no changes in any of these characters.

BA$_2$ and TMO are smooth strains, serologically identical.

Both contain the Kauffmann somatic antigen IV and not the antigen V which Kauffmann (1936) believes was mistaken for a virulence antigen by Felix and Pitt (1936). BA₂ is highly virulent while TMO kills a majority of mice only in large doses. All the known methods of demonstrating the Vi antigen of *Eberthella typhosa* have failed in our hands to give evidence of a Vi antigen in BA₂. There is no significant difference in the toxicity for mice of killed cultures of BA₂ and TMO. They can be distinguished culturally, however, by the fact that 18-hour broth cultures of BA₂ regularly show a larger number of bacilli than similar cultures of TMO, and BA₂ has a somewhat smaller average cell size than TMO. Strain RZ1 is a rough variant of TMO; it kills mice only in massive doses. Strain V47D is also rough; it was derived from a smooth strain possessing both somatic antigens. V47D kills mice in slightly smaller doses than RZ1.

Most of the mice used in these experiments were Swiss mice obtained directly from one breeder who maintains a large inbred stock; a few were raised in this laboratory from the same stock. A strain of more resistant mice was obtained by crossing a Swiss doe with a wild brown buck and breeding the offspring with Swiss mice. The white offspring from this mating were inbred for two generations to provide the Swiss-Brown mice used for virulence tests. A third lot of mice was obtained from the laboratory stock of the New York State Department of Health[1] and will be referred to as Albany mice. Swiss and Swiss-Brown mice were inoculated when they were six to eight weeks old and weighed 18 to 24 grams. The Albany mice weighed 22 to 26 grams when injected. No spontaneous mouse typhoid infection has been detected in any of these animals.

Virulence tests were performed by inoculating groups of 25 mice intraperitoneally. Inoculated mice were kept in individual cages. All mice dying within 28 days were autopsied and cultures were made from the spleen and blood. If these cultures were negative, as rarely happened, the mouse was considered a survivor. Mice which survived 28 days were killed and cultured in the same manner.

[1] Supplied through the courtesy of Dr. M. B. Kirkbride.

The inocula consisted of suspensions of 18-hour agar cultures, standardized either by measuring the volume of packed cells after centrifugation or by adjusting the opacity to compare with that of a barium sulphate standard; the suspensions were diluted in 0.85 per cent NaCl to contain the desired number of live bacilli in 0.5 ml. All inocula were checked by pouring plates immediately before injection. The average deviation from the calculated dose was 10 per cent and never exceeded 30 per cent.

Blood for culture was in most cases taken from the tails of mice in capillary pipettes selected to deliver 38 to 42 drops to the cubic centimeter. When more than 2 or 3 drops of blood were desired, the mice were anesthetized and bled from the heart with a 25-gauge needle. This procedure, however, was not often used, unless the mouse was to be sacrificed, because of the risk of killing the animal.

Peritoneal exudate for culture or smear was obtained by inserting a short, sharp capillary pipette through the abdominal wall. A preliminary comparison of smears stained with Wright's stain and preparations stained supravitally with neutral red showed nearly identical differential counts. Since the former method permitted a more accurate determination of phagocytosis and provided permanent preparations, it was adopted.

RELATION OF DOSE TO MORTALITY AND TO SURVIVAL TIME

Although numerous investigators have recorded experiments in which mice and rats were injected intraperitoneally with various doses of micro-organisms of the paratyphoid-enteritidis group, the work of Lockhart (1926) and of Topley (1927b) affords the most extensive data on the relation of dose to mortality. Using large numbers of mice Lockhart found that mortality increased gradually from 31 to 91 per cent as the dose of *Salmonella typhimurium* was increased a million fold. From this he concluded that large variations in dosage had relatively little effect on the percentage of deaths and that it was impossible to determine accurately the minimal lethal dose of such cultures with any reasonable number of animals. Wilson (1930), using a much shorter range of dosage, found that within the dose range

of about 30 to 200 live virulent bacilli there was no correlation between the number of *Salmonella typhimurium* injected and the mortality percentage of the injected mice.

It soon became apparent in our work that, with some strains at least, a small increase in dose in a certain range produced a marked increase in mortality.

Results of virulence titrations

The results of virulence titrations are shown in table 1. In some instances more than one test was done with the same dose. Not only are the three-day mortality and total mortality percentages recorded but also the mean survival times, which include the mice killed at the end of 28 days as well as those which died before that time. For purposes of comparison the mortality figures taken from Lockhart's paper (1926) are included. Lockhart's experiments were terminated at 14 days and therefore his mean survival times cannot be compared with ours. Although we have confirmed the experience of others (Lockhart, 1926) that the majority of deaths occur before the 14th day, we have observed a sufficient number of delayed deaths to warrant extension of the observation period. For example, in the test with a dose of 7 bacilli of strain BA₂, the 14-day mortality was 72 per cent and the 28-day mortality 84 per cent. Delayed deaths were particularly frequent in tests with vaccinated mice, none of which is included here.

Text-figure 1 is the graphic presentation of the mortality data in Table 1 and shows the curves or *characteristics* (Trevan, 1927) obtained by plotting per cent mortality against the logarithms of the number of bacilli injected. Since doses of 100 or more of BA₂ in Swiss mice left only an occasional survivor, the curve is nearly horizontal. The range in which a decrease in the dose of this strain might reduce the mortality includes doses which are too small to measure accurately. The result of the test with a dose of 7 bacilli suggests that one bacillus is capable, in some instances, of killing a mouse.

In sharp contrast to the BA₂-Swiss curve, the curve for TMO in the same strain of mice shows an insignificant number of deaths

with a dose of 500,000 or less, but between 500,000 and 1,000,000 the curve is almost perpendicular.   The slope of the curve for

TABLE 1

Mortality percentages and mean survival times of mice after intraperitoneal injection of increasing doses of Salmonella typhimurium

STRAIN OR SOURCE OF DATA	MICE	DOSE	NUMBER OF ANIMALS	THREE-DAY MORTALITY	TOTAL MORTALITY	MEAN SURVIVAL TIME LIMITED TO 28 DAYS
				per cent	*per cent*	
BA₂, smooth, virulent	Swiss	7	25	0	84	13.04
		10^2	25	0	100	8.08
		10^3	10×25	0.4	98.5	7.18
		10^5	25	0	100	5.23
		10^7	25	84	100	2.76
	Swiss-Brown cross	10	25	0	40	21.20
		10^3	2×25	0	62	18.00
		10^5	2×25	0	92	9.12
		10^6	25	0	100	5.48
TMO, smooth, low virulence	Swiss	10^2	25	0	0	28.0
		10^3	25	0	4	27.12
		10^5	2×25	0	2	27.72
		2×10^5	25	0	4	27.12
		5×10^5	25	0	8	26.44
		8×10^5	2×25	0	40	20.96
		10^6	2×25	0	90	7.32
		10^7	2×25	58	98	4.24
		5×10^7	25	92	100	2.56
	Albany	10	2×25	0	38	19.36
		10^3	25	4	92	11.0
		10^5	25	0	100	9.0
		10^7	25	24	100	3.80
RZ1, rough, avirulent	Swiss	10^7	25	0	0	28.0
		10^8	3×25	0	9.3	25.92
		5×10^8	25	68	80	7.48
Lockhart (1926)		10	400	5	30.75	
		10^3	400	16	59.75	
		10^5	400	16	70.00	
		10^7	400	58.5	90.75	

RZ1 is similar to that of TMO although larger doses of RZ1 were required to kill a majority of mice.
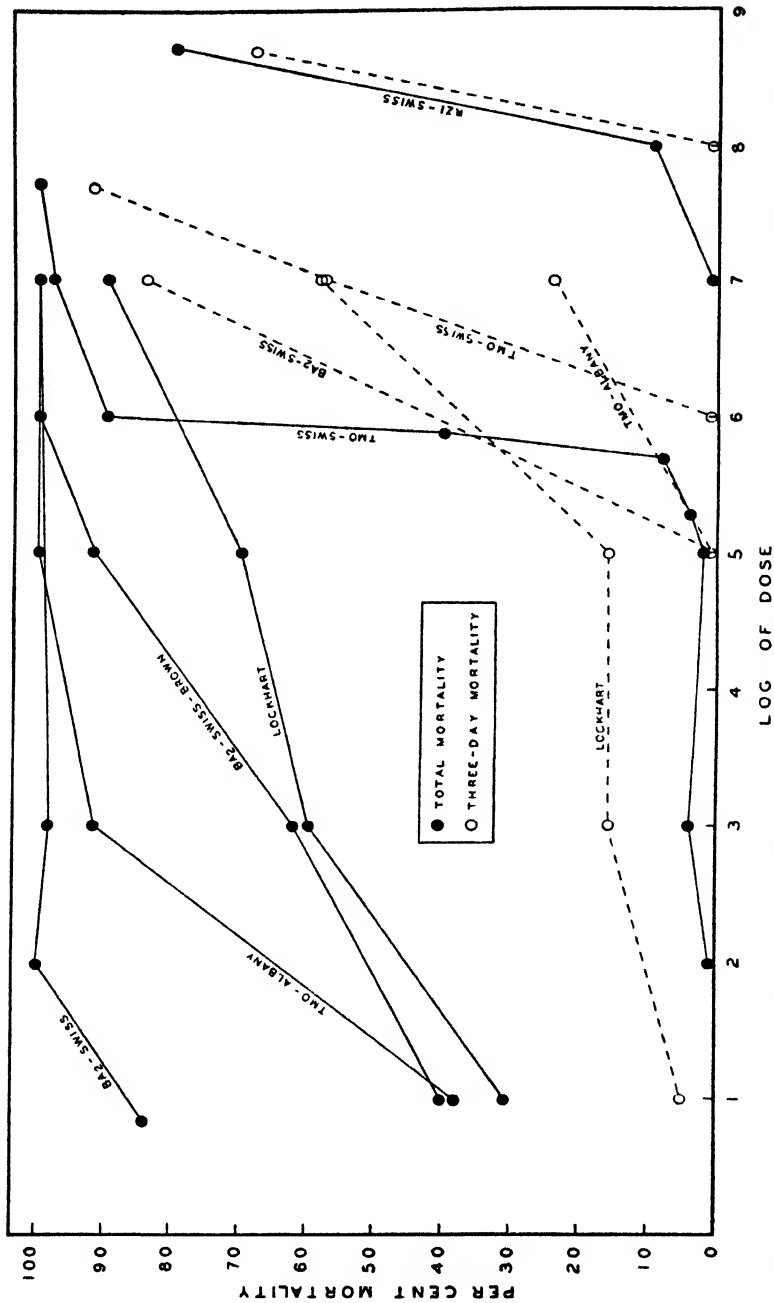
TEXT-FIG. 1. MORTALITY OF MICE INJECTED INTRAPERITONEALLY WITH INCREASING DOSES OF SALMONELLA TYPHIMURIUM

In comparing the *characteristics* of our cultures with the curve drawn from Lockhart's figures it is seen that the latter show no range of doses in which there is an abrupt change from very low to very high mortality. At a dose of about 1,000,000 this curve crosses the TMO-Swiss curve and all doses used caused a considerable number of deaths.

Examination of the total mortality and 3-day mortality curves for BA_2, TMO, and RZ1, using Swiss mice, shows that the less the virulence of the strain, the nearer the 2 curves approach each other due to the greater proportion of acute deaths with lethal doses of the less virulent strains. RZ1 did not kill until the dose approached the range where it was large enough to be primarily toxic while BA_2 in doses too small to be toxic was able to grow in the mouse and cause death after a period of incubation. Although Lockhart's strain killed fewer mice than BA_2, he observed 3-day deaths with doses which in our experiments gave none.

In an attempt to duplicate the gradual slope of Lockhart's curve 5 other smooth cultures of *Salmonella typhimurium* were tested in Swiss mice. Two of these were comparable in virulence to BA_2 while 3 were less virulent and caused a sharp increase in mortality in about the same dose range as TMO. None showed a gradual increase in mortality such as Lockhart observed.

When TMO was tested in Albany mice which proved to be more susceptible than the Swiss, the curve approached the BA_2-Swiss curve (text-fig. 1). The Swiss-Brown mice injected with doses of BA_2 increasing from 10 to 1,000,000 showed an increase in mortality from 40 to 100 per cent giving a curve which approached the slope of the Lockhart curve. The rate of decrease in survival time with increasing doses (table 1) was in all instances similar to the rate of increase in mortality. The mean survival time figures are of value, however, in that they show the effect of increasing the dose beyond the point where 100 per cent mortality is obtained.

Although a complete titration of the virulence of BA_2 in Albany mice was not performed, testing these mice with a dose of 1,000 BA_2 illustrated a possible source of misleading results in virulence

tests.   As shown in table 2, when BA$_2$ and TMO were tested with doses of 1,000 in Albany mice neither the difference in mortality nor in survival time was significant.   There can be no doubt of the difference in virulence of the 2 strains, however, when tested in the less susceptible Swiss mice.

Discussion

The rate at which mortality increases with an increase in dose might be affected by two factors, namely, the bacteria and the mice.   We have attempted to explain the difference in the slope of Lockhart's curve and the curve for TMO in Swiss mice by analyzing both factors.   If a strain possessed low infective

TABLE 2

Mortality percentages and mean survival times of 2 strains of mice after intraperitoneal injection of BA$_2$ and TMO

MICE	STRAIN	DOSE	PER CENT MORTALITY	MEAN SURVIVAL TIME LIMITED TO 28 DAYS
Albany.................... {	BA$_2$	10^3	100	8.20
	TMO	10^3	92	9.68
Swiss............... ... .. {	BA$_2$	10^3	98.5	7.18
	TMO	10^3	4	27.12

capacity and produced fatal infection only when given in large doses, one would expect to obtain curves similar to those of TMO and RZ1 in Swiss mice.   On the other hand, a highly infective strain which killed a considerable number of mice with small doses might give a curve similar to Lockhart's or, if still more virulent, a curve similar to that of BA$_2$ in Swiss mice.

If we examine the 3-day mortality curves from the same experiments, a difference in the infective capacities of the strains seems to be a less plausible determinant of the slope of the curves. The 3-day mortality curves for BA$_2$, TMO, and RZ1 in Swiss mice are nearly parallel regardless of the capacity of the strains to infect.   Lockhart's 3-day mortality curve, however, shows a more gradual slope due to some early deaths with small doses. This suggests that some of Lockhart's mice were extremely

susceptible, while others died only after an incubation period of more than 3 days.

The more probable explanation for the difference in the slope of dose-mortality curves is based upon differences in the test animals. It is obvious that the greater the degree of uniformity in susceptibility of the test animals, the steeper the *characteristics*. A group of animals all of which had exactly the same degree of susceptibility would all either die or survive after the injection of any particular dose. Trevan (1927) pointed out that inbred animals which tend toward uniformity give steeper *characteristics* than heterogeneous animals. The mortality figures which Irwin (1929) recorded for rats which had been brother and sister mated for 60 generations show a rapid increase in mortality with an increase in the dose of *Salmonella enteritidis*. Lockhart's mice were selected at random from the stock of several different breeders while the Swiss mice used by us were from an inbred strain. Although Topley's (1927a) analysis of Lockhart's experiments shows a variation in the average susceptibility of lots of 20 mice which was no greater than would be expected on the basis of the normal curve of error, one cannot exclude the probability that the greater uniformity of our inbred Swiss mice accounts for the observed difference in the dose-mortality curves.

The virulence titration of BA_2 in the Swiss-Brown mice illustrates the effect of the susceptibility of the test animals upon the shape of the dose-mortality curve. These mice probably had not been inbred for a sufficient number of generations to become uniform with respect to susceptibility. We do not know to what extent the Albany mice were inbred but the steepness of the first portion of the curve suggests a fairly uniform susceptibility.

Webster (1923) and Pritchett (1925) found differences in the susceptibility of different strains of mice to *per os* infection with *Salmonella typhimurium*. Lockhart (1926) considered the differences recorded by Pritchett not significant and in his own work found no difference in the resistance of mice from 3 sources. Our results indicate that there may be wide differences in the average susceptibility of different strains of mice. This difference, however, may not be equally apparent with all doses since the

relation of dose to mortality is not the same in all strains of mice and dose-mortality curves which are not parallel must either converge or cross.

Our results also demonstrate (table 2) that 2 strains of *Salmonella typhimurium* which appear to be about equally virulent when tested in highly susceptible mice may show a large difference when tested in more resistant animals. In other words, by using test animals of greater resistance one does not decrease the mortality at the same rate for all strains. Topley (1927b) emphasized the necessity of knowing the *characteristic* of the microorganism before selecting the optimal differential dose. We have shown that it is also important to know the resistance of the particular strain of mice used in the test.

Summary

1. When Swiss mice from a uniform inbred stock were inoculated intraperitoneally with increasing doses of strains of *Salmonella typhimurium* covering a wide range of virulence, the increase in mortality was found to be much more rapid within a certain range than the work of other investigators had indicated. Evidence is discussed which seems to indicate that the slope of dose-mortality curves is dependent more upon the degree of uniformity of the test animals with respect to susceptibility than upon the infective capacities of the test cultures.

2. It is not possible to plot a dose-mortality curve that will apply to all strains of *Salmonella typhimurium* tested under various circumstances.

3. Large differences in the virulence of strains may not be apparent if they are tested in highly susceptible mice over only a narrow range of doses.

4. Recording the mean survival time of a group of test animals gives valuable information regarding virulence and host susceptibility not revealed by total mortality figures alone.

FACTORS INFLUENCING THE COURSE OF INFECTION

It has been shown repeatedly that the course of experimental infections is in part dependent upon the virulence of the micro-

organisms used. Such experiments as those of Bull (1914) and those of Wright (1927) demonstrate that virulence affects the rate of removal of streptococci and pneumococci from the circulating blood after intravenous injection. With respect to mouse typhoid infection, Webster (1923a and b) has shown that after oral administration blood stream invasion is more likely to occur if highly virulent strains are used than if the strains are of low virulence. More recently Ørskov *et al.* (1928a and b, 1930) and Jensen (1929) in studying the rôle of the reticulo-endothelial tissues in various paratyphoid infections in normal and immunized mice recorded the course of events following *per os* infection with graded doses of several strains. They reported observations on a strain of *Salmonella typhimurium* (Breslau) of reduced virulence which retained the invasive capacity of the original virulent culture, but they do not state whether or not any other changes accompanied this partial loss of virulence.

Preliminary observations on the course of infection

In order to observe the sequence of events in the course of infection with the highly virulent strain, BA₂, and with the avirulent strain, V47D, mice were inoculated intraperitoneally in triplicate with doses ranging from 1,000 to 100,000,000 live bacilli. At intervals the condition of the mice was noted, plates poured with samples of blood and peritoneal exudate, and smears made from the exudate.

With BA₂ it was found that blood cultures might be positive within 5 minutes after inoculation. The number of bacilli that reached the blood during the first hour increased with an increase in the number injected. This initial bacteriemia usually had decreased or disappeared before the end of the first 6 hours, unless the dose was overwhelming (1,000,000); it recurred or increased again after several hours or days depending on the size of the dose. A similar temporary decrease in the number of bacilli in the peritoneal exudate was not observed; the number remained nearly constant for a period, the duration of which was in general inversely proportional to the size of the

dose.   An increase in the number of bacilli in the peritoneum could usually be demonstrated slightly in advance of the rise in the bacteriemia.   It was frequently noted that mice which appeared quite well had as many as 400,000 virulent bacilli per ml. of blood.   Mice became sick only after the severe bacteriemia had persisted for a number of hours.   Increasing the dose of BA_2 from 1,000 to 100,000 increased the number of bacilli in the blood during the first hour and shortened the survival time from about 7 days to about 5 days.   With both doses there was a period of lag (Lockhart, 1926) lasting 3 or 4 days before the final, invariably fatal, bacteriemia.   Increasing the dose to 100,000,000 shortened the survival time by eliminating the lag.   The course of the infection during the last day before death was the same whether the dose was large or small.

Comparing this course of events with that which followed the injection of the relatively avirulent strain, V47D, it was found that V47D invaded the blood stream during the first hour in much smaller numbers, although rapid invasion of the blood did occur with doses too small to kill.   In some instances when organisms were never demonstrated in the blood, positive spleen cultures were obtained 28 days after injection.   Differences in the reaction of mice to the injection of BA_2 and V47D were much less apparent with the large doses.

Examination of peritoneal exudates showed that the number of cells present at various times was in accordance with the well known course of the acute inflammatory reaction in the peritoneum (Topley and Wilson, 1936), namely, an initial decrease followed by a rapid rise beginning about 1 hour after inoculation. The range, before inoculation, of differential counts on peritoneal fluids of 25 of the mice used in these experiments was comparable to that recorded by Goodner and Miller (1935b) for mice of 18 to 24 grams.   The proportion of polymorphonuclear neutrophils present before inoculation was never over 1 per cent and no correlation was observed between the response of the polymorphonuclear cells and the outcome of the disease.   Nor was there any correlation in our data, obtained from mice of approximately the same age and weight, between the relative proportions

of monocytes and lymphocytes and the course of infection as was observed with pneumococcus infection (Goodner and Miller, 1935a and b). The proportion of polymorphonuclear cells increased to as much as 60 per cent 6 hours after injection, the increase being in general proportional to the number of bacilli injected and to the virulence of the culture.

Phagocytosis in the peritoneal exudates was noted but except after the injection of very large doses or during the terminal stages of infection, numbers of bacilli in the exudates were too small to permit phagocytic counts.

A few observations on mice which had been vaccinated with acetone-dried bacilli indicated that the partial immunity resulting from vaccination with the smooth somatic antigen reduced the initial bacteriemia in mice infected with smooth virulent bacilli. A similar reduction in the number of rough avirulent bacilli reaching the blood stream was not observed in mice vaccinated with the rough somatic antigen.   These facts are in accord with previous observations (Mackenzie, Fitzgerald and Pike, 1935) on the immunizing properties of smooth and rough strains of *Salmonella typhimurium.*

Distribution and survival of the bacilli in the tissues of infected mice

In order to trace the distribution throughout the body of intra-peritoneally injected cultures of different degrees of virulence, experiments were performed in which roughly quantitative cultures were made from tissues at intervals after inoculation. Three series of 13 mice each were inoculated with approximately 100,000 BA₂, TMO, and V47D respectively.   At intervals as shown in table 2, from 1 to 3 mice of each series were killed with ether and examined as follows using sterile instruments for each procedure:

The hair on the chest and abdomen of the mouse was soaked down with alcohol and the thorax was opened without incising the abdominal wall or diaphragm.   After removing as much blood as possible from the heart, the abdominal skin was laid open without incising the peritoneum.   The right inguinal lymph node was removed and emulsified by macerating it in saline on

a sterile glass slide with the aid of forceps.   The entire node was mixed with melted agar and plated.   The peritoneum was then opened and thoroughly washed with 1 ml. of 0.85 per cent NaCl; dilutions of this suspension were plated.   The mesenteric lymph nodes were removed and cultured in the same manner as the inguinal node.   The spleen and a portion of the liver weighing about 0.5 grams were plunged into boiling water for 1 or 2 seconds and then macerated for plating by pushing them through fine copper gauze in the bottom of a small syringe.

The results of the 3 experiments are shown in table 3.   Because of the nature of the experiment the figures are only roughly quantitative; and the unequal susceptibility of the mice decreases the significance of observations on a single mouse.   The occurrence of negative spleen and liver cultures in the presence of positive cultures from the peritoneal exudate indicates that dipping in boiling water usually sufficed to destroy the surface bacteria. Some, but certainly not all, of the bacteria cultured from the liver, spleen, and nodes were present in the blood contained in those tissues; in many cases bacteria were cultured from tissues when blood cultures were negative.

With regard to the numbers of bacteria in the peritoneal exudates, the figures in table 3 indicate that BA_2 and the less virulent TMO survived about equally well during the first 24 hours.   By the end of 3 days the numbers of BA_2 had begun to increase. With TMO there was little change up to 7 days and mice killed at 21 days showed negative cultures.   In the mice inoculated with V47D, there was a more rapid reduction in the numbers of bacilli in the peritoneal cavity although cultures were positive, in one instance, after 21 days.

This observation on the relative ability of the 3 strains to survive in the peritoneum was confirmed by inoculating groups of 10 mice with 100,000 bacilli of each strain.   Plate counts made on the peritoneal exudates aspirated from each mouse at 1, 8, and 24 hours after inoculation were comparable to those recorded in table 3.

The counts of the bacilli in the blood showed approximately the same numbers of BA_2 and of TMO during the first 24 hours,

TABLE 3

Distribution and survival of Salmonella typhimurium in the tissues of mice inoculated with 100,000 bacilli

STRAIN	TIME AFTER INOCULATION	COLONIES DEVELOPING FROM					
		Peritoneal exudate	0.025 ml. of blood	0.5 grams of liver	Spleen	Mesenteric lymph node	Inguinal lymph node
BA₂	1 hour	20,000	40	90	70	35	0
	3 hours	40,000	150	1,760	1,500	530	4
	6 hours	65,000	36	360	175	800	4
		25,000	30	130	1,200	900	0
	24 hours	560	2	5,000	10,000	540	1
		3,300	1	1,000	6,700	2,000	35
	3 days	70,000	16	10,000	45,000	12,000	1,000
		450,000	120	100,000	200,000	50,000	2,600
	7 days	No survivors					
TMO	1 hour	27,000	110	300	1,200	65	7
	3 hours	11,000	65	320	1,200	200	3
	6 hours	5,600	6	10	400	30	3
		25,000	23	240	600	200	0
	24 hours	700	2	120	320	350	2
		2,000	1	250	900	1,300	0
	3 days	640	0	20	360	700	0
		14,500	2	140	12	7,700	280
	7 days	900	0	50	220	1,700	0
		2,000	0	70	1,000	1,700	480
	21 days	0	0	0	0	1	0
		0	0	1	1	23	0
	1 death						
V47D	1 hour	1,400	0	0	0	1	0
	3 hours	0	0	0	0	0	0
	6 hours	20	0	0	0	0	0
		60	0	0	0	0	0
	24 hours	35	0	0	0	0	0
		10	0	0	0	0	0
	3 days	75	0	6	25	30	0
		90	0	0	0	1	5
	7 days	1,300	0	13	11	220	0
		280	0	100	225	25	1
	21 days	0	0	0	85	1	0
		0	0	0	70	41	0
		8	0	120	975	4	6
	No deaths						

out at the end of 3 days the counts on BA₂ mice were rising while those on TMO mice were declining. With V47D the blood cultures were all negative although in preliminary experiments an occasional colony was obtained from the blood of mice receiving this dose (100,000 bacilli) of V47D.

With respect to cultures from liver, spleen, and lymph nodes the first marked difference between BA₂ and TMO appeared in 24 hours. The increase in numbers of BA₂ in the liver and spleen preceded the increase in numbers in the blood. V47D did not invade the organs until the third day, but after 21 days V47D had survived in the organs in greater numbers than TMO. This capacity of V47D to survive in the spleen was noted in previous virulence tests in which 36 or 75 per cent of 48 mice surviving the injection of 1,000 V47D for 28 days showed positive spleen cultures, while 10 or 24 per cent of 41 mice surviving the same dose of TMO were harboring *Salmonella typhimurium* in their spleens at the end of 28 days. This difference is five times its standard error.

Bacteriemia following intraperitoneal inoculation

From the data recorded in table 3 it was evident that after intraperitoneal inoculation V47D invaded the blood stream in much smaller numbers than BA₂ and TMO but between BA₂ and TMO no significant difference was apparent. Consequently, more mice were inoculated with BA₂ and TMO in doses ranging from 1,000 to 1,000,000. Blood cultures were taken from the tail at intervals as shown in table 4 and text-figure 2. In general with both cultures the bacteriemia showed an increase in the numbers of bacilli in the circulating blood up to 6 hours after inoculation, followed by a decrease between the 6th and 24th hours. Exceptions to this were seen with mice which received 1,000,000 BA₂ and with mice which received both cultures in a dose of 1,000 bacilli; with these doses the phase with decreasing numbers of bacilli in the blood was eliminated—injection of the smaller of these two doses was followed by a lag period of about 48 hours. The critical period in the infection was between the 24th and 48th hours; during this period the number of bacteria

in the blood of mice destined to die usually began to increase and, in mice destined to survive, to decrease.   The average difference in the numbers of BA_2 and of TMO in the blood in the early phases

TABLE 4

Bacteriemia following intraperitoneal injection of Salmonella typhimurium

DOSE	NUM-BER OF MICE	TIME AFTER INJECTION	COLONIES PER 0.025 ML. OF BLOOD			
			BA₂		TMO	
			Average	Range	Average	Range
1,000	6	15–20 minutes	1	0–1	1	0–1
		6 hours	1	0–3	1	0–1
		24 hours	1.3	0–3	1.3	0–4
		48 hours	2.5	1–5	2.7	0–9
		72 hours	33	10–110	2.0	1–4
10,000	6	20–30 minutes	8	4–16	3.3	0–5
		6 hours	18	3–54	5.3	1–11
		24 hours	14	3–38	1.7	1–4
		48 hours	14	1–55	5	0–13
		72 hours	654	10–3,500	7	0–28
75,000	20	15–50 minutes	26	4–56	18	2–52
		6 hours	56	0–143	29	0–120
		24 hours	5.3	0–57	1.3	0–7
		48 hours				
		72 hours	803	0–9,000	1	0–6
100,000	6	45–50 minutes	53	31–88	59	33–83
		6 hours	89	27–137	37	8–70
		24 hours	3.6	0–6	0.5	1–5
		48 hours	64	13–130	1.7	1–5
		72 hours	3,000	270–10,000	1.2	0–2
1,000,000	6	45–50 minutes	100	29–158	63	22–120
		6 hours	1,300	118–3,000	790	185–2,250
		24 hours	2,150	7–6,600	26	3–80
		48 hours	9,500	100–20,000	77	6–210
		72 hours			260	12–1,100

of the bacteriemia was in most instances surprisingly small, and with all doses, some mice injected with TMO showed higher counts than some of the BA_2 mice.   The dose of 75,000 was given to groups of 20 mice and the average difference in counts up to

24 hours seemed, when analyzed statistically, probably not significant. In general, the smaller the dose, the longer the period that colony counts of the blood of mice receiving the two cultures remained approximately the same. Thus, with a dose of 1,000 the curves separate at 48 hours, with a dose of 75,000 at 24 hours, and with a dose of 1,000,000 at 6 hours.
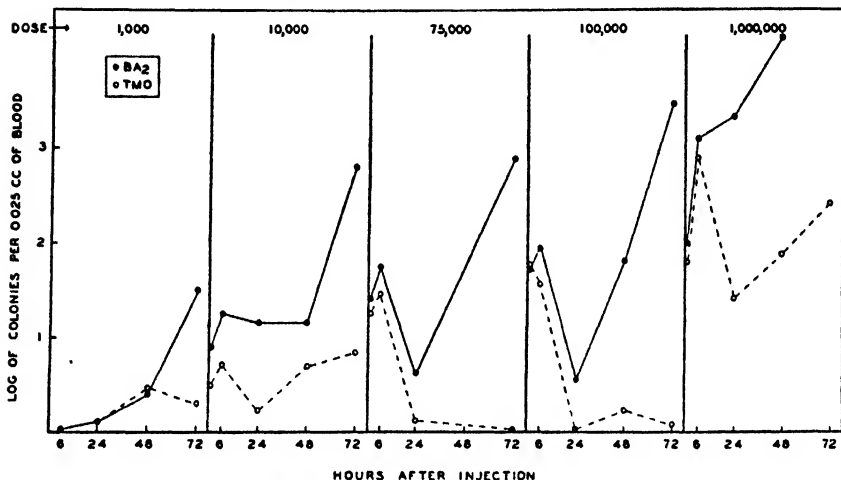
TEXT-FIG. 2. GRAPHIC REPRESENTATION OF DATA IN TABLE 4 SHOWING NUMBER OF BACTERIA IN THE BLOOD OF MICE AT INTERVALS AFTER THE INTRA-PERITONEAL INJECTION OF INCREASING DOSES OF SALMONELLA TYPHIMURIUM

Susceptibility to phagocytosis

Attempts to demonstrate differences in the susceptibility of the 3 strains to phagocytosis in peritoneal exudates *in vivo* were not successful for two reasons. First, the exudates of mice with no preliminary preparation contained for several hours after the injection of a bacterial suspension so few cells that phagocytic counts were not possible. In the second place, although the above difficulty could be obviated by preparing the mice with intraperitoneal injections of heat-killed staphylococci 24 hours before the injection of the test culture, the smallest dose of bacteria which insured a sufficient number of bacteria in smears for counting was about 10,000,000. With a dose of this size

BA₂ and TMO were phagocytized equally well, and this was to be expected since with such a large dose as this the percentage mortalities and mean survival times of mice injected with these two strains are nearly equal. V47D, on the other hand, in a dose of 10,000,000, disappeared so rapidly from the exudates of prepared mice that in smears made 20 minutes after injection only an occasional free or ingested bacillus could be found.

For the determination of phagocytosis *in vitro* the most satisfactory method of obtaining leucocytes was found to be the production of sterile peritoneal exudates by injecting 0.5 ml. of a 5 per cent suspension of aleuronat. After 24 hours, mice were bled from the heart and the peritoneal exudates from 2 mice were suspended in 1 ml. of 0.85 per cent NaCl containing heparin 1:500. The same leucocyte suspension was used for all 3 cultures in each experiment. Bacterial suspensions were made from 18-hour cultures on agar slants and were standardized in a Petroff-Hausser counting chamber. For the tests 0.05 ml. of bacterial suspension, 0.05 ml. of fresh mouse serum, and 0.1 ml. of leucocyte suspension were combined in small tubes and incubated at 37°C. Samples for smears were removed at intervals. The tubes were shaken before the removal of each sample.

The phagocytosis of the 3 strains *in vitro* is shown in table 5. A total of 900 polymorphonuclear leucocytes was counted in the 3 experiments with each culture. The results taken as a whole show that the order of increasing susceptibility to phagocytosis corresponds to the order of decreasing virulence. But these small differences in susceptibility to phagocytosis are in striking contrast to the large differences in virulence. Although small differences in susceptibility to phagocytosis are nearly constant, we are inclined to believe that they are inadequate to explain the differences in virulence.

Intracellular digestion was not observed with any of the strains in smears made up to 30 minutes, neither do the figures recorded in table 5 show a decrease in the number of intracellular bacilli after incubation up to 40 minutes. By using a formalin-killed suspension to prevent multiplication, intracellular lysis could be demonstrated after several hours incubation, but differences in

the susceptibility of the 3 strains to lysis were not observed. When parallel tests were made with a Vi strain of *Eberthella typhosa*, it was found that the typhoid bacillus was more susceptible to intracellular lysis but lysis was not as rapid in mouse leu-

TABLE 5

Phagocytosis of Salmonella typhimurium strains by mouse polymorphonuclear leucocytes in vitro

NUMBER OF BACILLI IN TEST	TIME OF INCUBATION	PER CENT OF CELLS INGESTING			BACTERIA INGESTED BY 100 CELLS		
		BA₂	TMO	V47D	BA₂	TMO	V47D
	minutes						
I. 25,000,000	5	25	22	36	28	28	58
live bacilli	15	85	82	89	174	200	282
	30	98	96	99	437	362	469
II. 25,000,000	5	15	27	51	17	36	74
live bacilli	15	92	94	96	220	259	396
	30	94	99	100	425	522	626
III. 5,000,000 live	10	12	18	11	12	19	13
bacilli	20	19	27	31	23	31	34
	40	41	46	45	53	64	66
Total for 900 cells		53.4	56.8	62.0	1,389	1,521	2,020

TABLE 6

Phagocytosis in the omentum 15 minutes after the intraperitoneal injection of about 10,000,000 live bacilli

STRAIN	PER CENT OF MACROPHAGES INGESTING	NUMBER OF BACILLI INGESTED BY 100 MACROPHAGES
BA₂..............................	7	12
TMO..............	20	32
V47D...........................	34	115

cocytes as Lucké *et al.* (1933) found it to be in rabbit leucocytes. We found no greater differences in the relative susceptibility of these strains to phagocytosis by mononuclear cells in the exudates than to polymophronuclear phagocytes.

Phagocytosis in the omentum was studied by killing mice at intervals after the intraperitoneal injection of bacterial suspen-

sion, removing the omentums and spreading them out on glass slides.   The thin films of omentum, after drying, were stained with Wright's stain and examined under the microscope.   In the omentum of a normal mouse, polymorphonuclear leucocytes are rare but macrophages in considerable numbers are present, singly and in small clusters.   Fifteen minutes after inoculation there was some increase in the number of macrophages but polymorphonuclear phagocytes did not gather until about 45 minutes later. Table 6 shows typical results of phagocytic counts on omentums removed 15 minutes after the intraperitoneal injection of about 10,000,000 live bacilli.   TMO was phagocytized in somewhat larger numbers than BA₂, and V47D in much larger numbers than either of the smooth strains.

Discussion

Although bacteria have been administered by the intraperitoneal route in numerous studies of mouse typhoid infection, Amoss (1922), Webster (1923a), Topley (1920), and Ørskov, Jensen, and Kobayashi (1928a) infected mice by the natural route, the gastrointestinal tract.   It has been argued that the natural portal of entry should always be used to avoid drawing erroneous conclusions from artificial conditions.   On the other hand, *per os* administration introduces fortuitous and uncontrollable variables.   Although the use of a stomach tube makes possible more accurate dosage than when bacilli are fed to the animal, the number of bacteria which pass the intestinal barrier is still to a large degree dependent upon chance.   The disease in mice is a septicemia and not in any important sense an infection of the gastrointestinal tract; furthermore, it has been shown (Ørskov *et al.*, 1928a and 1930) that once these micro-organisms gain entrance to the blood stream the course of the disease is the same regardless of the portal of entry.   Also it is clear that in such experiments as these it is important to control dosage as accurately as possible and that the effective dosage is the number of bacilli which gain parenteral rather than *per os* entrance; hence it would seem that intraperitoneal injection eliminates certain uncontrollable variables and gives rise to a disease having all the

important characteristics of the natural *per os* infection. It is apparent too that strains which show high virulence when administered *per os* also show high virulence when injected intraperitoneally or subcutaneously; the converse with respect to avirulent strains is also true (Ørskov and Lassen, 1930). Similarly, Webster (1933) has shown that mice that are naturally resistant to enteritidis infection *per os* are also resistant if injected intraperitoneally or intravenously.

Two of the essential components of virulence, one may reasonably conclude, are invasiveness and at least some toxigenicity. Without these characters there would be avirulence, but it does not follow, as our results demonstrate, that virulence arises from a combination of invasiveness and toxigenicity; a strain may possess these characters and be relatively avirulent. The work of Ørskov and Lassen (1930) suggests that an analysis of the virulence of mouse typhoid bacilli should also take into consideration the ability of the strains to multiply in the animal body.

In the strains we have studied the rôle of toxigenicity is probably of secondary importance. Although toxic substances can be extracted from *Salmonella typhimurium* (Grasset *et al.*, 1935; Martin, 1934; Boivin *et al.*, 1933) their potency is relatively low and the presence of an effective concentration of these substances in the body is dependent upon extensive multiplication of the bacteria. No difference has been observed in the toxicity of BA_2 and TMO (Mackenzie, Pike, and Swinney, 1940).

Analysis of presumptive components of virulence of the strains studied here shows that after intraperitoneal injection BA_2 and TMO readily pass the primary barriers against invasion and in approximately equal numbers. BA_2 is perhaps slightly more invasive than TMO. In resistance to phagocytosis by mouse leucocytes *in vitro* the difference between the two strains is again small but in favor of the virulent strain. To what extent the large difference in virulence between these two strains may be ascribed to the small average differences in invasiveness and resistance to phagocytosis is uncertain. We are inclined to believe that these differences are too small to justify the conclu-

sion that either invasiveness or resistance to phagocytosis is a major determinant of the virulence of *Salmonella typhimurium* for mice. The most marked difference observed in the course of the infection with these 2 strains was in the ability of the more virulent strain to multiply in the mouse. With small and medium sized doses the survival and multiplication in the mouse of the smooth strain of high virulence and the smooth strain of low virulence showed very slight differences during the first 24 or 48 hours. After this initial lag, the duration of which depends on the size of the dose, the virulent strain, though present in the tissues in small numbers, survives, multiplies and kills the mouse while the strain of low virulence is unable to increase in numbers, usually dies out, and permits the mouse to survive. It appears that what we have observed is at least a partial separation of invasiveness and capacity to multiply in the host. BA$_2$ possesses both of these characters, TMO only invasiveness.

Summary

In an attempt to identify the character or characters upon which virulence for mice of *Salmonella typhimurium* depends, we have studied the distribution, survival, and multiplication in the mouse of strains covering a wide range of virulence. A smooth strain of high virulence and a smooth strain of low virulence, indistinguishable in cultural, serological, immunizing, and toxigenic characters, did not differ significantly in invasiveness and resistance to phagocytosis. An unmistakable difference, however, was found in the ability of the virulent strain to multiply in the host.

Compared to the smooth strains, an avirulent rough strain was found to be much less invasive and to exhibit very little capacity to multiply in the mouse. It did, however, somewhat paradoxically, possess greater capacity to survive in the spleen.

The results of our experiments seem to justify the conclusion that sustained ability to multiply in the host is not necessarily an accompaniment of invasiveness; in strains of low virulence, invasiveness may be as highly developed as in strains of high virulence.

Under the conditions of these experiments, therefore, growth energy manifested by ability to multiply in the environment provided by the host appears to be a major determinant of virulence.

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VIRULENCE OF SALMONELLA TYPHIMURIUM

II. Studies of the Polysaccharide Antigens of Virulent and Avirulent Strains

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In a previous communication (Pike and Mackenzie, 1940) we have recorded the results of studies which were part of an attempt to identify and evaluate the factors which determine the virulence of *Salmonella typhimurium* for mice. First, it was shown that quantitative determinations of the virulence of *Salmonella typhimurium*, if they are to have validity, should include careful consideration of the dosage factor and of the degree of resistance of the mice to this infection: with some strains of this micro-organism a small increment in dosage causes a large increase in percentage mortality; measurement of virulence with highly susceptible mice may fail to reveal differences which are clearly demonstrated when adequate numbers of more resistant mice are used. Furthermore, it was shown that invasiveness and resistance to phagocytosis are at most minor determinants of virulence, and that sustained ability to multiply in the tissues of the host is the character which differentiates most unmistakably the virulent from the avirulent strain.

That the immunizing capacity and the toxicity of several members of the typhoid-paratyphoid group of bacteria depend upon polysaccharide antigens has been reported by Boivin, Mesrobeanu, and Mesrobeanu (1933), Raistrick and Topley (1934) and Felton and Wakeman (1937); these studies have not, however, revealed whether or not these antigens are also important as determinants of virulence. Previous studies (Mackenzie, Fitzgerald, and Pike, 1935), in which intact bacilli treated with

alcohol were used, indicated that the virulence of *Salmonella typhimurium* did not depend upon the smooth somatic antigen. In these experiments no attempt was made to isolate the polysaccharide antigens or to determine their significance as determinants of virulence. Even though there is considerable evidence (Boivin, Mesrobeanu, and Mesrobeanu, 1933; Raistrick and Topley, 1934) which seems to indicate that the principal constitutents of the smooth somatic antigen are polysaccharides, it seemed necessary in attempting to analyze the virulence of this species, to study the properties of the isolated and partially purified polysaccharide antigens.

Cultures

Two smooth cultures of *Salmonella typhimurium*, BA₂ and TMO, and two rough cultures, V47D and RZ1, which we have used in this work have been described in detail elsewhere (Mackenzie, Fitzgerald, and Pike, 1935; Pike and Mackenzie, 1940). The colonies formed by V47D and RZ1 have grown progressively less rough over a period of 4 years but this change has not been accompanied by changes in any of the other characteristics of rough cultures. Observations on the virulence, the immunizing power, the invasiveness, and the resistance to phagocytosis of these strains have been recorded (Mackenzie, Fitzgerald, and Pike, 1935; Pike and Mackenzie, 1940).

Methods

Mass cultures were grown on beef infusion agar pH 7.6 in 15 cm. petri dishes. The 18- to 20-hour growth was collected by flooding the agar with sterile distilled water and suspending the bacteria by scraping the surface of the agar with a platinum wire. The heavy suspensions thus obtained were filtered through absorbent cotton to remove particles of agar.

Three methods were employed in the preparation of antigenic material:

1. The method of Raistrick and Topley (1934). An equal volume of acetone was added to suspensions of bacteria in distilled

water in large centrifuge tubes.   After standing at room tempera-
ture for 1 hour the tubes were centrifuged, the supernatant fluid
discarded and the sedimented bacteria emulsified in acetone.
After standing at 37°C. over night the tubes were again cen-
trifuged, the supernatant fluid discarded, the sediment washed
twice with small amounts of acetone and dried.   The tryptic
digestion and fractionation of the dried bacilli were carried out
exactly as described by Raistrick and Topley (1934).   At this
point it became apparent that the yield of each fraction was
relatively small.   Since the fractions which Raistrick and
Topley precipitated by concentrations of alcohol from 50 to 68
per cent differed so little and since they subsequently used only
the 68 per cent alcohol precipitation (Topley *et al*, 1937), we
combined the 68/50, 68/60, and 68/68 fractions and reprecipi-
tated this material with 68 per cent alcohol.   This material will
be referred to as the Raistrick-Topley antigen.

*2. The method of Boivin et al.   (1933, 1934a and b, 1935a
and b).*   To the tryptic digest, prepared as in the Raistrick-
Topley method above, trichloracetic acid was added to a con-
centration of N/4 and extraction carried out at 4°C. for 3 hours.
The mixture was then centrifuged and the opalescent supernatant
fluid placed in dialyzing sacs made with a 5 per cent solution of
collodion in glacial acetic acid.   The material was dialysed for
48 hours against running water and, after acidulation, for 24
hours against distilled water.   The contents of the sacs were
then removed, acidulated, and precipitated with 5 volumes of
alcohol.   The precipitate was washed with alcohol and ether and
dried.   This material constituted the Boivin antigen.

3. Extraction with distilled water at 60°C.   This method was
essentially that described by Smith (1938) for the preparation of
toxic extracts from *Eberthella typhosa*.   Similar methods have
previously been used for extracting toxic and antigenic substances
by Pick (1902, 1912), Weil and Felix (1920), Fukuhara and Ando
(1913), Chase (1931), White (1931), and Landsteiner and Levine
(1932).   Bacterial suspensions were prepared as before except
that cold distilled water was used and the suspension was kept in
an ice bath.   This cold suspension was centrifuged, the super-

natant removed, and the bacteria resuspended in distilled water. After heating in a water bath for 4 hours at 59 to 60°C., the suspension was again centrifuged and the supernatant extract filtered through a Berkfeld N candle.   The filtrate was precipitated with 5 volumes of alcohol, the white precipitate washed

TABLE 1

Yield and chemical analysis of antigenic fractions prepared from Salmonella typhimurium

PREPARATION	STRAIN	YIELD	QUALITATIVE CHEMICAL ANALYSIS			QUANTITATIVE CHEMICAL ANALYSIS						
			Protein	Amino acid	Carbohydrate	Glucose	C	H	N	S	P	Residue
		per cent				per cent	per cent	per cent	per cent	per cent	per cent	per cent
Raistrick-Topley	BA₂, smooth high virulence	8	−	+	+	40.0	37.8	6.3	5.0	0.5	3.5	14.4
	TMO, smooth low virulence	8	−	+	+	39.5	38.5	6.3	6.7	0.4	3.1	11.3
	V47D, rough very low virulence	1	−	+	+	23.5	35.2	6.2	6.4	0.6	4.9	21.0
	RZ1, rough avirulent	1	−	+	+	24.0	20.2	4.7	4.9	0.5	9.6	41.2
Boivin	BA₂	9	−	+	+	45.1	44.5	6.6	5.5	0.5	0.9	7.0
	TMO	8	−	+	+	51.9	39.4	7.5	4.7	0.6	1.2	3.5
	V47D	1	−	+	+	44.1						
Aqueous extract	BA₂	7	−	+	+	43.9						
	TMO	6	−	+	{+}	37.8						
	V47D	4.5	+	+	{+}	16.1						
	RZ1	4.5	+	+	+	17.4						

with alcohol and ether and dried.   This preparation will be called the aqueous extract.

With one exception, we have applied these methods to the preparation of antigens from all 4 strains and have obtained yields as shown in table 1.   The yield from each strain is expressed as a percentage of the weight of acetone-dried bacilli. Since the aqueous extracts were prepared from bacteria which

had never been dried, the weight of dried bacteria in this case was estimated from previously acquired knowledge of the average amount of growth of these strains. The amount of growth varied considerably among different strains, but for any one strain it was fairly constant. Although the yield was much higher from smooth strains (8 to 9 per cent) than from rough strains (1 per cent) by both the Raistrick-Topley and Boivin methods, the 2 methods showed little difference in the yield from any one culture. Precipitation of the aqueous extracts of smooth cultures yielded slightly less material (6 to 7 per cent) than precipitation of material obtained by the other methods; the yield from rough cultures by aqueous extraction was relatively high (4.5 per cent).

Although the solubility and appearance of the dried antigens was dependent to some extent upon the speed of drying, definite differences in solubility in 0.85 per cent NaCl were noted. For use as stock solutions the antigens were usually dissolved in a concentration of 0.4 per cent. In this concentration the aqueous extracts of smooth cultures dissolved readily forming opalescent solutions. The Boivin smooth antigens dissolved more rapidly than the Raistrick-Topley antigens but both formed stable solutions of about the same concentration. The antigens prepared from rough cultures, with the exception of the Boivin antigen from V47D, were less soluble and formed, even in distilled water, much less stable suspensions than the smooth antigens. Neither of the RZ1 antigens formed a stable suspension.

THE CHEMICAL PROPERTIES OF THE ANTIGENS

The results of qualitative and quantitative chemical analyses are shown in table 1.

Exton and Esbach tests for protein were performed on solutions or suspensions containing at least 0.1 per cent of the antigens and were negative on all preparations except the two aqueous extracts of rough cultures. Positive biuret, Millon, and xanthoproteic tests indicated the presence of peptide linkages, phenolic bodies (probably tyrosine), and phenyl groups in all preparations. The presence of carbohydrate groups in all the antigens was shown by positive Molisch tests.

Reducing substances expressed as glucose were determined by the Hagedorn-Jensen method after hydrolysis by boiling for 1 hour in normal HCl. Content of reducing sugars was of the same order for all the preparations from smooth strains, 38 to 52 per cent, but there was a suggestion that antigens with slightly higher reducing sugar content were obtained by the Boivin method. The antigens from rough strains tended to have a lower content of reducing substances than smooth antigens prepared by the same methods.

The elementary analysis of the Raistrick-Topley antigens from strains BA_2 and TMO agreed closely with the results reported by Raistrick and Topley (1934). The low carbon and high phosphorus content of the Raistrick-Topley antigen from the rough strain, RZ1, and the relatively large amount of residue in antigens from rough cultures may be significant; the low phosphorus and low residue in the Boivin antigens were probably results of dialysis.

TOXICITY AND ANTITOXIC IMMUNITY

The toxicity of various preparations was determined by intraperitoneal inoculation of mice weighing 20 to 23 grams. The concentration of antigen in the inoculum was such that the desired dose was contained in 0.5 ml. of 0.85 per cent NaCl. The results are shown in table 2. The Raistrick-Topley antigens from strains BA_2 and TMO were perhaps slightly more toxic than equal weights of whole acetone-dried bacilli.

The capacity of the Boivin preparations from strain BA_2 to induce antitoxic immunity was tested by immunizing mice with 7 injections at 3-day intervals increasing from an initial dose of 0.01 mgm. to a final dose of 0.1 mgm. The test dose was given 1 week after the last immunizing dose. The results, shown in table 3, indicate that the immunizing procedure protected against only about 1 minimal lethal dose. Other observations on the increased resistance of mice surviving toxicity tests were comparable to the results of this experiment.

TABLE 2

Numbers of deaths in mice following the intraperitoneal injection of various amounts of acetone-dried bacilli and polysaccharide antigens

STRAIN	PREPARATION	AMOUNTS OF ACETONE-DRIED BACILLI AND POLYSACCHARIDE ANTIGENS							
		10 mgm.	5 mgm.	2.5 mgm.	2.0 mgm.	1.0 mgm.	0.5 mgm.	0.25 mgm.	0.1 mgm.
BA₂	Acetone-dried bacilli				3/5*	8/25			
	Raistrick-Topley				10/10	27/35			
	Boivin						9/10	0/10	
	Aqueous extract						5/5	10/10	0/5
TMO	Acetone-dried bacilli				5/5	13/25			
	Raistrick-Topley				10/10	20/35	0/10		
	Aqueous extract						5/5	6/10	
V47D	Acetone-dried bacilli	4/5	5/5		0/10	0/15			
	Raistrick-Topley		2/2	2/2		0/2			
RZ1	Acetone-dried bacilli	5/5	5/5		4/10				
	Raistrick-Topley		0/2						

* In this table the numerator of each fraction records the number of deaths and the denominator the number of mice tested.

TABLE 3

Deaths among normal and immunized mice following the injection of the Boivin antigen prepared from BA₂

DOSE	NUMBER OF MICE	DEATHS	
		Immunised	Controls
mgm.			
2.0	5	5	5
1.0	5	3	5
0.5	5	1	4

PRODUCTION OF IMMUNITY TO INFECTION

In order to determine the most effective doses of the antigenic fractions for the production of active immunity to infection with virulent *Salmonella typhimurium*, groups of 10 mice were vaccinated intraperitoneally. The mice in each group received 2 injections of the same dose with an interval of 7 days between injections. The doses employed were 0.1, 0.01, 0.001, and 0.0001

mgm.   The antigens used were the Raistrick-Topley antigens
from strains BA_2 and TMO and the Boivin antigen from BA_2.
Two weeks after the second immunizing injection, half the mice
were injected intraperitoneally with 1,000 and half with 100,000
BA_2.   The degree of protection was found to be roughly pro-
portional to the size of the immunizing doses but the dose of 0.1
mgm. showed little advantage over 0.01 mgm.   Since 0.1 mgm.
approached the lethal dose of the aqueous extract of BA_2, 0.01
mgm. was employed in subsequent comparative immunity tests.

TABLE 4

Immunity of mice vaccinated with polysaccharide antigens of Salmonella
typhimurium

VACCINATED WITH		NUMBER OF MICE	SURVIVING 28 DAYS	MEAN TIME TO DEATH	MEAN SURVIVAL TIME	COM-PLETELY RESISTANT
Strain	Preparation					
			per cent	*days*	*days*	*per cent*
BA_2	Acetone-dried bacilli	25	40	16.67	21.20	4
	Raistrick-Topley	25	28	14.83	18.12	16
	Boivin	25	56	14.64	22.48	56
	Aqueous extract	25	0	11.68	11.68	0
TMO	Acetone-dried bacilli	25	40	16.07	20.84	40
	Raistrick-Topley	25	48	14.62	21.04	40
	Aqueous extract	25	4	14.04	14.60	4
Unvaccinated controls		100	3	8.02	8.59	0

Table 4 shows the results of immunity tests on mice vaccinated
with polysaccharide antigens from BA_2 and TMO.   Groups of
25 mice were vaccinated with two doses of 0.01 mgm. a week
apart and tested by the intraperitoneal injection one week later
of 1,000 live bacilli of strain BA_2.   We have recorded the per-
centage of mice surviving for 28 days, the mean time to death of
the mice which succumbed, the mean survival time limited to
28 days, and the percentage of completely resistant mice as
indicated by negative cultures from the blood and spleen after
28 days.

Of 100 unvaccinated controls only 3 per cent survived.   There
was no significant difference in the protection induced by the

acetone-dried bacilli and the Raistrick-Topley antigens of either culture. The Boivin antigen from BA₂ gave a little more protection than the Raistrick-Topley antigen: the difference between 56 and 28 per cent survivors is probably, but not certainly, statistically significant. The aqueous extract antigens conferred practically no protection on the mice although they slightly prolonged the mean time to death.

SEROLOGICAL ANALYSIS

The serological analysis was chiefly concerned with: (a) an analysis of the antigenic properties of the polysaccharide from strains BA₂ and TMO, (b) the similarities and dissimilarities of the polysaccharide antigens from smooth and rough strains, (c) a comparison of polysaccharide antigens prepared by different methods from the same culture, and (d) the relation of these antigens to somatic agglutinogens. In attempting to analyze the antigenic properties of the polysaccharide antigens, we have studied agglutinogenesis, precipitinogenesis, and specific absorptive capacities for agglutinins and precipitins. Although the analysis did not include antigens prepared by all 3 methods from all 4 strains, it did serve as a basis for comparing the Raistrick-Topley antigens from the 4 cultures and the BA₂ antigens prepared by the 3 methods.

Rabbits were immunized with 7 injections at 5 day intervals starting with 0.015 mgm. of the antigens from smooth cultures and 0.15 mgm. of the antigens from rough cultures and increasing the dose to 0.7 mgm. The small initial dose of smooth antigens was necessary because of the toxicity of these antigens for rabbits.

Somatic agglutinins were determined with suspensions of 24-hour agar cultures heated for 4 hours at 56°C. in 95 per cent alcohol. The antigens for detecting flagellar agglutinins were 24-hour broth cultures to which 0.2 per cent formalin was added.

Precipitin tests were performed by stratifying dilutions of antigen in 0.2 ml. amounts on 0.2 ml. of serum dilution, usually 1 to 10. The tubes were placed in a 50°C. water bath, observed for ring formation after 20 minutes, shaken, and allowed to remain at 50°C. for 4 hours. Final readings were made after stand-

ing over night at room temperature. When it was desired to determine the optimal proportions of antigen and antibody, the tests were observed at frequent intervals until the first tube to show flocculation had been recorded. Although rings appeared within a few minutes, flocculation usually did not appear for an hour or more.

In absorbing sera with polysaccharide antigens it was found that one absorption at the optimal ratio, or with an excess of antigen, sufficed to remove detectable homologous precipitins but in no case did one absorption of a low dilution of serum completely remove homologous somatic agglutinins. This apparent complete removal of precipitins was presumably due to the fact that a relatively high concentration of antibody was necessary to cause flocculation. A single absorption lowered the titer of precipitin to a concentration at which the test failed to reveal the presence of precipitin. Precipitation did not take place if the sera were diluted beyond 1 to 40. Complete removal of homologous agglutinins was obtained, however, by repeated absorptions.

Since temperature has been shown to affect the precipitation of antibody by pneumococcus SSS (Heidelberger and Kendall, 1935; Brown, 1935), absorptions were performed at 4°C., at 50°C. and at several intermediate temperatures. It was found that absorption at the low temperatures was no greater than at 50°C. Flocculation took place much less rapidly at low temperature.

A serological analysis of the Raistrick-Topley fractions of all 4 strains is shown in table 5. None of these preparations produced flagellar agglutinins. The antigens from smooth strains gave rise to O agglutinins and to precipitins for the smooth polysaccharide antigen. Reciprocal absorption of agglutinins with the antigens of BA_2 and TMO was complete—further evidence of the serological identity of these 2 strains.

Antigens from smooth strains and those from rough strains were shown to be serologically distinct by the absence of cross agglutination and cross absorption of agglutinins. The study of the rough antigens was limited by the small quantities obtained

and by the insolubility of the RZ1 antigen. A suspension of this antigen which was sufficiently stable to use in precipitin tests was never obtained. Like the smooth antigens, the V47D preparation was shown to be a complete antigen; it gave rise to agglutinins and precipitins and specifically precipitated the antibody. Although the RZ1 antigen failed, after prolonged immunization, to produce agglutinins in a rabbit, it absorbed agglutinins from V47D serum. Whether this antigen is a haptene or whether the failure to produce antibodies was due to the rabbit is not known.

Tests for the inhibition of O agglutination by the polysaccharide antigens were performed by incubating together decreas-

TABLE 5

Antigenic analysis of the polysaccharide antigens prepared by the Raistrick-Topley method from smooth and rough strains of Salmonella typhimurium

STRAIN	SOMATIC AGGLUTININS								PRECIPITINS			
	Titers of agglutinins for				Titers for homologous strains after absorption with polysaccharide antigens from				Smallest amount of antigen giving flocculation with 0.02 ml. serum prepared with			
	BA₂	TMO	V47D	RZ1	BA₂	TMO	V47D	RZ1	BA₂	TMO	V47D	RZ1
									mgm.	*mgm.*	*mgm.*	*mgm.*
BA₂...	1280	1280	0	0	0	0	1280	1280	0.006	0.006	None	None
TMO .	1280	1280	0	0	0	0	1280	1280	0.006	0.012	None	None
V47D. .	0	0	640	320	640	640	0	0	None	None	0.003	
RZ1.. .	0	0	0	0								

ing amounts of antigen and a constant amount of serum for 4 hours at 50°C. Any precipitate which formed was allowed to settle over night. The supernatant fluid was decanted; to it was added an equal quantity of alcoholic antigen, and agglutination allowed to take place in the usual manner. The amount of serum used was such that the final dilution corresponded to the highest dilution that would give complete agglutination. The results are summarized in table 6.

The data show that the amounts of polysaccharide antigen from strains BA₂ and TMO required completely to inhibit agglutination of either BA₂ or TMO by sera prepared with live bacilli and with polysaccharide antigens of these strains differed

only slightly. The differences recorded were only those represented in each instance by one tube in a series of antigen dilutions. It should also be noted that both the smooth and the rough polysaccharide antigens completely inhibited agglutination by homologous sera produced with live bacilli. This would seem to be additional evidence that the polysaccharide antigen is the somatic antigen upon which O agglutination depends.

TABLE 6

Inhibition of somatic agglutination by polysaccharide antigens prepared by the Raistrick-Topley method

SERUM PRODUCED WITH	ANTIGEN IN AGGLUTINATION TEST	SMALLEST AMOUNT OF ANTIGEN REQUIRED TO INHIBIT AGGLUTINATION COMPLETELY*		
		BA₂	TMO	V47D
		mgm.	*mgm.*	*mgm.*
BA₂—Live culture	BA₂	0.2	0.2	
	TMO	0.2	0.2	
BA₂—Raistrick-Topley.	BA₂	0.2	0.4	
	TMO	0.4	0.8	
TMO—Live culture	BA₂	0.4	0.4	
	TMO	0.8	0.8	
TMO—Raistrick-Topley..	BA₂	0.2	0.4	
	TMO	0.8	0.8	
V47D—Live culture	V47D			0.2

* Serum dilution in the inhibition tests was the highest dilution of serum giving complete agglutination.

At the outset it was thought that possibly the optimal proportions technic of Dean and Webb (1926) might be used to detect differences in the antigen content of different fractions. The amounts of BA₂ and TMO fractions prepared by all 3 methods which gave optimal flocculation in various sera were determined. It was found that the relationship of fractions as determined in one serum did not necessarily hold for another serum. For example, the aqueous extract antigen of BA₂ gave optimal flocculation in an antiserum for the polysaccharide prepared from BA₂

by the Raistrick-Topley method in one quarter of the optimal amount required for the homologous antigen, while in an antiserum for the polysaccharide prepared from BA₂ by the Boivin method these same fractions gave optimal flocculation in equal amounts. These discrepancies may have been due to differences in the antigenic constitution of the fractions, but the meaning of the results was not sufficiently clear to justify any conclusions.

The serological relations of the three BA₂ fractions to each other were studied by reciprocal absorption of agglutinins. All three completely absorbed somatic agglutinins from antisera prepared with each of the fractions and from sera prepared by the injection of live bacilli.

As in the case of the polysaccharide antigens prepared from BA₂ by the Raistrick-Topley method, the Boivin preparation gave rise to somatic agglutinins only, but the aqueous extract preparation also induced the formation of flagellar agglutinins in high titer.

Since it has been shown (Sordelli and Mayer, 1931; Morgan, 1936) that polysaccharide antigens prepared from bacteria grown on agar media may be contaminated with a non-specific polysaccharide from the agar itself, the sera used in this work were examined for the presence of antibodies for constituents of the medium. The antigen was prepared by precipitating a concentrated aqueous extract of beef infusion agar with acetone, dissolving the precipitate in 0.85 per cent NaCl, and reprecipitating with alcohol. This precipitate dissolved in a small amount of saline was tested against antisera for polysaccharides prepared by the 3 methods. Antisera for the Raistrick-Topley and Boivin polysaccharides gave no reaction with this antigen but an antiserum for the aqueous extract preparation gave a precipitin rereaction only with a concentrated solution of the agar antigen. This antigen was evidently present in the polysaccharide prepared by the aqueous extract method in sufficient amount to induce the production of antibodies; but it could not have introduced an error in the results of the serological reactions with this fraction because of the high dilutions at which these reactions took place. The nonspecific antigen was not studied further except to show

that it probably originated from either the beef or the peptone since no acetone-precipitable substances were present in an extract of pure agar.

Both Boivin and Mesrobeanu (1935a) and Raistrick and Topley (1934) have indicated their belief that they were dealing with the same substance in the antigenic material which they respectively have prepared. The former (Boivin and Mesrobeanu, 1934c), however, stated that their material contained a higher concentration of toxic and antigenic substance because of the lower nitrogen content and higher toxicity. We have tried both methods on the same strains: although a slight difference was observed in toxicity, the nitrogen content of the two preparations was almost the same; the lower residue and much lower phosphorous, probably inorganic, of the Boivin preparations probably indicate greater purity. We found little difference in the time and labor involved in the two methods.

With regard to the presence of polysaccharide antigens in rough cultures of gram-negative bacilli reports disagree. White (1929) failed to obtain a soluble specific substance from rough Salmonellas by extraction with acetic acid but Furth and Landsteiner (1929) at about the same time reported the extraction of a haptene from rough *Salmonella typhimurium* by means of alkaline hypochlorite. Later, White (1931), using the alkaline method by which Meyer (1930) obtained a haptene from rough *Shigella dysenteriae*, confirmed the work of Furth and Landsteiner. The necessity for using an alkaline extraction was also shown by Meisel and Mikulaszek (1931). Boivin and Mesrobeanu during the course of their work failed in many attempts to obtain a complete antigen from rough strains of *Salmonella typhimurium;* Raistrick and Topley record no observations on rough strains. Using the methods of these last named investigators we have obtained antigenic polysaccharides from rough strains; and, at least one of the preparations, the polysaccharide prepared by the Raistrick-Topley method from V47D, was a complete antigen.

The toxicity of our Boivin and Raistrick-Topley fractions was

slightly less than that reported by others.   Boivin *et al.* (1935b) obtained antigens which regularly killed mice in doses of 0.1 mgm. while Martin's (1934) fractions prepared by the Raistrick-Topley method were lethal in doses of 0.5 mgm.   Since a dose of 0.5 mgm. in mice would be the equivalent of over 1 gram for man, this cannot be considered more than a low grade of toxicity. Although animals immunized with these toxic preparations show increased resistance to the toxin, the number of minimal lethal doses to which the animals become resistant is small: hence their antitoxinogenic properties are also of a low grade.

The comparison of antigens prepared by the simple method of aqueous extraction at 60°C. with those prepared by the Boivin and Raistrick-Topley methods revealed significant differences. The polysaccharides prepared by the aqueous extract method were the most toxic antigens but, in our hands, they were incapable of inducing active immunity to infection.

Our serological analyses of these antigens are in accord with previous observations of others which have seemed to justify the conclusion that these polysaccharide antigens are identical with, or at least constitute the serologically significant components of, the antigen upon which somatic agglutination depends. Polysaccharides prepared by all 3 methods induced in rabbits O agglutinins and specifically absorbed the O agglutinins from sera prepared with intact acetone-dried bacilli.   Thus the results of this study of the properties of the polysaccharide antigen prepared by the aqueous extract method indicate that the toxicity of these preparations and their capacity to induce in mice an active immunity are determined by different components. Furthermore, in view of the frequently demonstrated heat stability of the immunizing antigens of this group of bacteria, it seems improbable that heating (4 hours at 60°C.) destroyed the immunizing capacity of the aqueous extract polysaccharide without destroying its ability to produce somatic agglutinins.   These results also cast some doubt on the validity of conclusions which assign to the smooth somatic antigen a predominant rôle in the determination of active immunity.

The presence of protein in the aqueous extracts of rough strains

and its apparent absence from similar preparations from smooth strains is not easily explained. One might postulate either a difference in the solubility of the smooth and rough proteins or some physical difference in the rough and smooth cells which causes the smooth protein to escape extraction by the methods used.

The similarity in all respects of the polysaccharide antigens of the highly virulent strain BA₂ and those of the much less virulent strain TMO confirms our previous contention (Mackenzie, Fitzgerald, and Pike, 1935) in regard to the independence of virulence and the smooth somatic antigen. The polysaccharides of these 2 strains seem to be quantitatively and qualitatively the same. The unavoidable implication is that the virulence of these strains is largely determined by serologically inactive material.

CONCLUSIONS

1. Antigenic polysaccharides prepared from *Salmonella typhimurium* by the method of Raistrick and Topley and by that of Boivin and Mesrobeanu possess minor chemical and antigenic differences.

2. Polysaccharide antigens prepared by different methods may be serologically indistinguishable and yet show significant differences in toxicity and immunizing capacity; hence, these two characters are apparently not determined by the same components of the bacterial cell.

3. Qualitatively and quantitatively identical polysaccharide antigens were obtained from 2 smooth strains of *Salmonella typhimurium* although these strains have manifested a persistent and large difference in virulence for mice.

4. The smooth polysaccharide antigen of *Salmonella typhimurium* is not the major determinant of virulence.

5. Important determinants of virulence are serologically inactive.

6. A polysaccharide which is a complete antigen has been obtained from a rough culture of *Salmonella typhimurium;* it is serologically quite different from the polysaccharide of smooth strains.

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STUDIES ON HEMOLYTIC STREPTOCOCCI

VI. The Epidemicus Group

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In an earlier paper (1935) it was shown that, in hemolytic streptococci of Lancefield's group A which ferment lactose and salicin but not mannitol, there is some correlation between resistance to phage C/594 and ability to produce epidemics of septic sore throat. The present communication reports further studies on this resistant group of strains, as compared with the group of strains differentiated from it only by sensitivity to phage C/594, designated as *Streptococcus pyogenes* in the second paper of this series.

The group of resistant strains will here be designated the epidemicus group, deferring the description of the species *Streptococcus epidemicus* until further studies˙ have determined more clearly whether or not certain strains which differ more or less from the type strain should be included in the species. Definitely, however, the type strain of *S. epidemicus* must be the strain known in the literature as X40, isolated by Davis and Rosenow in 1912 from the spleen of a fatal human case during the Chicago epidemic of septic sore throat. Davis gave it the species name *epidemicus*. It is maintained in the American Type Culture Collection and designated as *S. epidemicus* No. 624 in the catalog. Our number is 890. Essential characters are as follows: It belongs to Lancefield's group A, ferments lactose and salicin but not mannitol, resists phage C/594, and belongs to Griffith's type 13.

Eighteen of Griffith's 30 serologic type strains were found to belong to Lancefield's group A and to produce the specified fer-

mentation reactions. Using the technique described in previous papers it was found that the 18 type strains may be divided into three groups according to sensitivity to phage C/594, as shown in table 1. One group includes strains sensitive to the filtrate, one includes strains resistant to the nascent phage, and an intermediary group includes two strains sensitive to the nascent phage, but resistant to the filtrate. (Sensitivity to the filtrate implies sensitivity to the nascent phage; resistance to the nascent phage implies resistance to the filtrate.)

Using the technique described by Pauli and Coburn, agglutinating serums were prepared with strains resistant to phage C/594 representing all of those Griffith types which include

TABLE 1

Sensitivity to phage C/594 of the 18 Griffith type strains which belong to group A and ferment lactose and salicin, but not mannitol

	GRIFFITH TYPE STRAINS	SENSITIVITY TO PHAGE C/594	
		Nascent	Filtrate
Sensitive...	2, 3, 4, 5, 8, 9, 11, 12, 24, 25, 26, 28	+	+
Intermediary	1, 29	+	−
Resistant....	13. 22. 27. 30	−	−

chiefly resistant strains; and with strains of type 1 of the intermediary group. A representative strain of type 29 of the intermediary group was not available when the serums were prepared.

As the study progressed it was found that certain sensitive strains of Griffith's types 9, 11 and 12 are serologically related to the resistant type strain 993 of type 27. The unabsorbed serum prepared with strain 993 agglutinated these three type strains to full titer. Therefore, serums were prepared with strains representing the three types, and cross reactions in the absorbed serums shown by some strains gave further evidence of the relationships.

The histories of the strains used for the preparation of serums are as follows:

Strains sensitive to phage C/594 filtrate.

No. 769, received from Dr. Griffith, labeled, "Symons, type 9."

No. 770, received from Dr. Griffith, labeled "Blackmore, type 11."

No. 771, received from Dr. Griffith labeled "S.F. 42, type 12."

No. 1324, received from Dr. Griffith, labeled "Matthews, type 25." (The reason for preparing a serum with this strain is given further on.)

Intermediary strains, sensitive to nascent phage but resistant to the filtrate.

No. 650, received from Dr. Griffith, labeled "S.F. 130, type 1."

No. 905, from a throat culture from a fatal human case in the Kingston, N. Y. (1930) epidemic of septic sore throat. It was previously studied by Wheeler, who reported that strains from the Kingston epidemic produced moist colonies and showed capsules typical of *S. epidemicus;* and that they produced toxin not entirely neutralized by "N.Y. 5" antitoxin. Griffith (1937) classified strain 905 in type 1.

Strains resistant to nascent phage:

Strain 890, previously referred to as the type strain of *S. epidemicus.*

Strain 989, received from Dr. Griffith, labeled "63T type 22."

Strain 993, received from Dr. Griffith, labeled "G 112 type 27."

Strain 1403, received from Dr. Griffith, labeled "Quinn, Middleborough, Type 30."

Strain 951, from a case of erysipelas, identified as type 30.

Strain 1321 was of interest in this study because it is a resistant epidemic strain which has been studied by other investigators. The details of its history were reported by Coburn and Pauli, who designated it the "Westwater" strain. They isolated it from a case of pharyngitis in an outbreak at the Pelham Home in which rheumatic attacks followed throat infection. Hooker studied it and found that it produced strong B toxin and weak A toxin. Griffith placed it in his type 25.

Because attempts to produce an agglutinating serum with strain 1321 were unsuccessful, a serum was prepared with strain 1324 of type 25 (its history is given above) although this, Griffith's type strain, differs from strain 1321 in being sensitive to C/594 filtrate.

Using Griffith's technique for rapid agglutination in absorbed serum, 212 strains were classified as to serologic type. Among them 69 were sensitive to C/594 filtrate, 60 were sensitive to the nascent phage but resistant to the filtrate, and 83 were resistant to the nascent phage.[1] The correlation between serologic types and resistance to phage C/594 is shown in table 2.

Among the 83 strains resistant to nascent phage C/594, 63.9 per cent were classified in five of Griffith's types: 13, 22, 25, 27

TABLE 2

Correlation between sensitivity to phage C/594 and agglutination typing in 212 strains

GRIFFITH TYPE	SENSITIVE TO FILTRATE		SENSITIVE TO NASCENT PHAGE; RESISTANT TO FILTRATE		RESISTANT TO NASCENT PHAGE	
	Number	Per cent	Number	Per cent	Number	Per cent
13	0		3		9	
22	0		0		2	
25	1	7.2	2	25.0	8	63.9
27	1		9		16	
30	3		1		18	
1	7	21.7	11	18.3	8	19.3
11	9		0		8	
Not agglutinated in the above 7 types	48	71.0	34	56.7	14	16.8
Total.............	69		60		83	

and 30; 19.3 per cent fell in the two types 1 and 11, leaving only 16.8 per cent of strains not agglutinated by those seven type serums. On the other hand among the 69 sensitive strains only 7.2 per cent were agglutinated by those five serums which agglutinated the majority of the resistant strains; 21.7 per cent were agglutinated by serums of types 1 and 11, leaving the majority of sensitive strains (71 per cent) untyped by the seven serums.

On further examination, the data presented in table 2 show that

[1] The comparative numbers of strains selected for this study to represent the various groups are not indicative of their prevalence as the cause of human disease.

more than half of the resistant strains (51.8 per cent) fell into three of Griffith's types, 13, 27 and 30, the serums of those types having been prepared with strains selected on account of resistance to the phage. Griffith's type 22, with the type strain resistant to phage C/594, was represented in our collection by only one other strain.

Approximately 10 per cent of all resistant strains fell in type 25, although as already stated, Griffith's type strain, No. 1324, used for the preparation of the agglutinating serum, was sensitive to the filtrate. In our collection of 11 strains belonging to type 25, strain 1324 was the only one sensitive to C/594 filtrate; 8 were resistant to the nascent phage; two were sensitive to the nascent phage but resistant to the filtrate.

Almost 10 per cent of resistant strains fell in type 1, the agglutinating serums having been prepared with strains which were sensitive to the nascent phage but resistant to the filtrate, a character common to many type 1 strains.

Although the strain of type 11 used for the preparation of agglutinating serum was sensitive to the filtrate, almost 10 per cent of resistant strains fell in this type, which, as already stated, was shown to be serologically related to type 27. On the other hand, an insignificant number of resistant strains were agglutinated by the serums of types 9 and 12, prepared against sensitive strains serologically related to type 27.

The correlation between resistance to phage C/594 and association with septic sore throat is shown in table 3.

In our collection there are group A strains, with the given fermentation characters, from 27 epidemics of septic sore throat which occurred in various parts of this country and in England, the earliest having been the Chicago epidemic of the winter 1911–12. Only one strain from a given epidemic was included in the table if all were found to be identical. From eight epidemics the strains were found to belong to two of Griffith's types, and from those epidemics two strains, representing the two types, were included in the table.

It was shown in an earlier publication (1936) that among the scarlet fever strains of our collection the majority of those having

the given fermentation characteristics are sensitive to nascent phage C/594. On the other hand, only 5 of the 35 septic sore throat strains (14.3 per cent) were found to be sensitive to the lytic filtrate; nine strains (25.7 per cent) were sensitive to the nascent phage but resistant to the filtrate, leaving the majority (60 per cent) completely resistant to the phage.

TABLE 3

Sensitivity to phage C/594, and agglutination typing of 35 septic sore throat strains from 27 epidemics*

SENSITIVITY TO PHAGE		NUMBER OF STRAINS	TYPE	NUMBER OF STRAINS OF THE GIVEN TYPE
Nascent	Filtrate			
+	+	5	9	1
			11	1
			27	2
			28†	1
+	–	9	Untyped	3
			1	2
			13	1
			27	2
			29†	1
–	–	21	Untyped	6
			1	2
			11	4
			13	4
			25	2
			30	3

* Nine duplicate strains, with all reactions identical with the type strain of the given epidemic, were omitted from this table.

† Griffith's type strains, not studied serologically by the writer.

Further observations made on the data presented in table 3 show that more than half of the 35 strains from septic sore throat epidemics (51.4 per cent) belonged to four of Griffith's types, 1, 11, 13 and 27, each of those types being represented by four or five strains.

DISCUSSION

In the previous paper of this series it was shown that distinctions made on the basis of sensitivity to phage B/563 were of an

order similar to the group distinctions made on the basis of precipitin reactions according to Lancefield's technique. In this paper it is shown that distinctions made on the basis of sensitivity to phage C/594 are of another order, some strains of group A being sensitive, and other strains being resistant. Studies reported in this paper were undertaken to determine whether relationships indicated by sensitivity to phage C/594 might be correlated with other characters which are of epidemiologic or immunologic significance.

The data show evidence of some correlation between distinctions made on the basis of sensitivity to phage C/594, and distinctions made on the basis of agglutinin absorption reactions. The data suggest, however, that antigenic structure and resistance to bacteriophage are linked together genetically, rather than that they depend on the same substance in the bacterial cell, because marked discrepancies in the relationship between the two properties may occur, as in the case of the sensitive strain 1324, belonging to type 25, a type which includes chiefly resistant strains.

The data confirm the observation previously reported, that there is some correlation between resistance to phage C/594 and ability to produce epidemics of septic sore throat. It is unknown to what extent such a reaction may be linked with those properties which determine protective responses in the host. Studies are in progress to show whether cross protection may be demonstrated between those Griffith types which are grouped together by the common character of resistance to phage C/594.

SUMMARY

Eighteen of Griffith's 30 type strains belong to Lancefield's group A and ferment lactose and salicin but not mannitol. Twelve are sensitive to phage C/594 filtrate; two are resistant to the filtrate but are sensitive to the nascent phage; four are resistant to the nascent phage.

Among 212 strains of Lancefield's group A which ferment lactose and salicin but not mannitol, the majority of strains resistant to phage C/594 (63.9 per cent) fell in Griffith's serologic types 13, 22, 25, 27 and 30. Only a few (7.2 per cent) strains sensitive to C/594 filtrate fell in those five serologic types.

Among 35 strains with the given characters from epidemics of septic sore throat, the majority (60 per cent) were resistant to nascent C/594 phage. Only 5 (14.3 per cent) were sensitive to the filtrate.

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STUDIES ON THE THERMAL SENSITIVITY OF MARINE BACTERIA[1]

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There are numerous reports (Benecke, 1933, Waksman, 1934, ZoBell, 1938) on the occurrence and importance of bacteria in the sea in different parts of the world but, unfortunately, the methods of investigation used by various workers have been so widely divergent that neither the qualitative nor the quantitative results are comparable. One of the greatest variables is the temperature to which the bacteria have been subjected, although Forster (1892), Drew (1910), Berkeley (1919), and others have emphasized the extreme thermal sensitivity of marine bacteria. In fact, due to a lack of appropriate refrigeration of water baths, incubators, and other facilities while working on a boat at sea, marine bacteria have been subjected to wide ranges of temperature. This paper is concerned with the effect that this may have upon the life processes and death of the bacteria.

Only those bacteria found in the sea which will grow in nutrient sea water media but not in corresponding freshwater media, or those which have been isolated from a marine environment at places remote from possibilities of terrigenous contamination, are regarded as marine species. This distinction is made to exclude bacteria of obviously terrestrial origin with which bays, estuaries and coastal waters are contaminated. While there may be an interchange of bacteria between the land and the sea (Burke,

1934) with certain bacteria common to both environments, the majority of those occurring under truly oceanic conditions are believed to be distinctive marine species (ZoBell and Feltham 1933).

EXPERIMENTAL METHODS

Samples of sea water for analysis were obtained by means of the bacteriological sampler described by ZoBell and Feltham (1934) and bottom deposits were obtained with a Trask coring tube (Hough, 1939). Radially central portions of the mud core were dissected out aseptically for bacteriological purposes. Most of the samples were analyzed shortly after collection although sometimes the urgency of other activities, rough weather or other adverse field conditions necessitated storage of the samples. Since earlier studies have revealed that the total number of bacteria in sea water (ZoBell and Anderson, 1936) as well as in mud samples (ZoBell, 1938) increases rapidly with storage, a change which is accompanied by a decrease in the number of different species discernible, the stored samples were held in the refrigerator at near 0°C. At this temperature the changes in the bacterial population are minimized, though not entirely prevented. There is no evidence that any species are killed or overgrown even after several days storage at 0°C., but many heat-sensitive species are destroyed by a few minutes exposure to temperatures exceeding 25°C. and the fastidious ones are soon crowded out when samples are stored at temperatures higher than 5°C. Prescott and Winslow (1931) have discussed the effect of storage upon water bacteria with particular reference to temperature.

Sterile sea water was used for dilution blanks. The nutrient media contained 0.2 per cent each of Bacto-peptone, proteose-peptone and beef extract and 0.002 per cent iron citrate in sea water using either 1.2 per cent agar or 10 per cent gelatin as solidifying agents. The reaction was adjusted to pH 7.6 with N/10 NaOH after autoclave sterilization. Unless otherwise stated, 10 ml. of medium was used to pour the plates and the plates were incubated for two weeks at 22°C. The colonies were counted with a Stewart colony counter using a 3.5 × engraver's lens.

POURING TEMPERATURE OF THE MEDIUM

Not infrequently nutrient agar is poured into inoculated plates before it has cooled to 42°C., below which temperature it begins to congeal. (Similar concentrations of agar congeal at a somewhat higher temperature in sea water than in freshwater.) In order to ascertain to what extent the temperature of melted agar influences plate counts, samples of sea water were plated with agar at temperatures ranging from 42° to 60°C. The medium was cooled to exactly the stated temperature in a water bath and then poured into 10 cm. Pyrex Petri dishes previously inoculated with 1.0 ml. of raw sea water.

TABLE 1

Relative number of colonies developing from sea water or marine mud when plated with nutrient agar at different temperatures, the plate counts being expressed as percentages of the average plate count on media poured at 42°C.

INOCULA	NUMBER OF SAMPLES	POURING TEMPERATURE OF AGAR				
		42°C.	45°C.	50°C.	55°C.	60°C.
		per cent	per cent	per cent	per cent	per cent
Sea water	14	100	95.8	89.4	34.2	17.5
Marine mud	9	100	93.4	82 1	26.9	11 4

More bacteria developed on the medium which was poured at 42°C. than on that poured at higher temperatures. Moreover, the agreement between duplicates was better when the agar was poured at the lower temperatures. The results obtained with 14 different samples of sea water and 9 of marine bottom deposits which were plated in duplicate with nutrient agar at different temperatures are summarized in table 1. The average plate counts are expressed as ratios on a basis of the 42°C. count being 100.

The data reveal that about 95 per cent as many bacteria form colonies when the agar is plated at 45°C. as when it is plated at 42°C. and successively smaller percentages at higher temperatures. However, the experiment fails to show what percentage of the bacteria may be rendered incapable of multiplication by pouring the agar at 42°C., a temperature which is considerably

in excess of that of their environment. Since agar begins to congeal at 42°C., gelatin was used to prepare a solid medium which could be plated at lower temperatures. Gelatin is not entirely satisfactory as a solidifying agent because it is liquified by the actively proteolytic bacteria which are abundant in the sea. Therefore, gelatin plates must be counted before the slower-growing bacteria have had time to develop into macroscopically visible colonies.

The nutrient gelatin was poured at temperatures ranging from 30° to 50°C. The plates were incubated at 12°C. for a week. The relative numbers of bacteria which developed on the medium poured at each temperature are shown in table 2 which gives the

TABLE 2

Relative number of colonies developing from sea water or marine mud when plated with nutrient gelatin poured at different temperatures, the plate counts being expressed as percentages of the average plate count on media poured at 30°C.

INOCULA	NUMBER OF SAMPLES	POURING TEMPERATURE OF GELATIN				
		30°C.	35°C.	40°C.	45°C.	50°C.
		per cent	per cent	per cent	per cent	per cent
Sea water.	12	100	98.6	96.5	87.5	76.2
Marine mud.	16	100	97.9	91.3	83.4	67.8

average of duplicate analyses on 12 samples of sea water and 16 of marine mud.

Approximately as many bacteria developed on the gelatin medium poured at 35°C. as on that poured at 30°C. and almost as many developed on the medium poured at 40°C. However, significantly fewer bacteria developed on the medium poured at higher temperatures. It is evident from these observations that while marine bacteria are extremely sensitive to heat, they are not sufficiently so to invalidate the use of nutrient agar poured at 40° to 42°C. for estimating the abundance of bacteria in marine materials since, at their best, plate counts detect only a small percentage of the bacterial population. It is obvious, though, that the medium should be cooled at least to 42°C. before pouring to insure comparable and maximum counts because even

at this temperature certain heat-sensitive species are inactivated. If, due to the exigencies of field conditions, media are poured at temperatures exceeding 50°C., more than half of the bacteria may fail to develop.   Drew (1910) reports that the bacteria from tropical waters around the Bahamas are very sensitive to temperatures as high as 40°C. and exposure at 45°C. causes the death of a large proportion of them.

In the foregoing experiments the plates themselves were at room temperature (21° to 22°C.) at the time the media were introduced.   It was found that when the plates were cooled on ice prior to the introduction of the medium, plating temperatures in excess of 42°C. were less injurious.   This is what might be expected because the introduced medium is cooled faster, thus subjecting the bacteria therein to the higher temperature for a shorter period of time.   As revealed by the results summarized in table 3, little or no practical advantage is gained by cooling the plates on ice when the medium itself is cooled to 42°C. before pouring but the beneficial effect of the use of ice is quite pronounced when the medium is poured at higher temperatures. The use of ice to hasten the cooling of the media might save a few heat-sensitive species from destruction but for practical purposes this procedure does not increase the plate count enough to offset the disadvantages of the extra work involved and the undesirable effects of the medium congealing in the cold plate before it has been evenly distributed (Green, 1936).

Other factors which influence the effect of the pouring temperature of the medium are the type of plate, the heat-conductivity of the table upon which the plate rests and the size of the inoculum.   The media are cooled faster by thick-walled Pyrex Petri dishes of high heat-holding capacity than by thin-walled ones, assuming that the dishes themselves are at a low temperature when the medium is introduced.   Similarly, the media are cooled faster when the plates are resting on a foundation of concrete, metal or soapstone than on one of wood.   Such factors influence the magnitude of plate counts and the agreement between duplicates.

Prescription bottles are quite widely used as a substitute for

Petri dishes for field studies.   One milliliter of the appropriately diluted sample is introduced directly into 15 ml. of melted nutrient agar in the bottles cooled to 40° to 42°C.   The inoculated bottles are then placed on their sides until the agar has solidified. In the initial experiments less than half as many colonies developed in prescription bottles inoculated by this procedure as when a similar medium was used in Petri dishes, probably because

TABLE 3

Relative number of colonies developing from sea water plated with nutrient agar poured at different temperatures into plates at different temperatures, the plate counts being expressed as percentages of the average plate count on media poured at 42°C. into dishes having a temperature of 21-22°C.

TEMPERATURE OF PLATE	POURING TEMPERATURE OF AGAR				
	42°C.	45°C.	50°C.	55°C.	60°C.
°C.	per cent	per cent	per cent	per cent	per cent
Near 0	108	103	95	68	51
21–22	100	97	86	41	16
30	79	72	61	36	14

TABLE 4

Relative number of colonies which developed from samples of sea water or mud after being held at the stated temperature for 10 minutes, the plate counts being expressed as percentages of the plate count of material held for ten minutes at 20°C.

INOCULA	NUMBER OF SAMPLES	EXPOSURE TEMPERATURE						
		20°C.	30°C.	40°C.	50°C.	60°C.	80°C.	100°C.
		per cent	per cent	per cent	per cent	per cent	per cent	per cent
Sea water........ ...	10	100	81.3	21.9	6.8	3.0	0.2	+
Marine mud . . .	10	100	68.5	18.3	10.3	5.2	0.7	+

the prescription bottles cool so slowly.   By immersing them to the neck for thirty seconds in ice water immediately after inoculating, almost as many colonies developed in prescription bottles as in Petri dishes.

Marine bacteria are not unique in their susceptibility to the pouring temperature of nutrient agar because the senior author found that the bacteria indigenous to Lake Mendota (a freshwater lake in Wisconsin) are similarly heat-sensitive.   Using the pre-

scription bottle technique, two to five times as many bacteria developed when the bottles were quickly cooled after inoculation by immersion in cold water as when they were merely permitted to cool on the table top, although in both cases the medium was cooled to 40° to 42°C. before inoculation.

The use of pre-solidified agar as advocated by Anderson and Stuart (1935) obviates the necessity of exposing the bacteria to the temperature of melted agar. While, statistically, the counts obtained by this procedure compare favorably with plate counts obtained by the conventional technique, there is a greater divergence of duplicates and many time-consuming precautions must be exercised to prepare satisfactory plates.

THERMAL DEATH POINT

The temperature tolerance of marine bacteria was determined using thermal death point technique. For this purpose 2.0 ml. portions of recently collected sea water or appropriately diluted mud samples were placed in 6 ml. serological tubes. The use of the small thin-walled tubes reduced to a minimum the time required to change the temperature of the contents. Pairs were immersed in water baths ranging in temperature from 20° to 100°C. After exactly ten minutes the tubes were transferred to ice water and 1.0 ml. of the heated suspension was spread uniformly over the surface of pre-solidified nutrient agar in Petri dishes. The plates were incubated at 12°C. for two weeks. Table 4 shows the average number of colonies which developed from samples of sea water and marine mud treated in this manner.

There is no evidence to suggest that any of the bacteria are injured by ten minutes exposure to a temperature of 20°C. regardless of the temperature of the environment from which they were obtained. However, about one-fourth of the bacteria were rendered incapable of multiplication in ten minutes at 30°C. and only one-fifth of them survived after being held for this period of time at 40°C. Direct microscopic observations of the material showed that the heat treatment had not merely caused the bacteria to clump together or to adhere to the walls of the sero-

logical tubes which would have reduced the plate counts. Moreover, as will be discussed below, the respiration of the bacteria was impaired by the heat treatment.

In general, the bacteria from bottom deposits were found to be somewhat more heat-sensitive than those occurring in sea water. A few heat-tolerant spore-forming bacteria were found in nearly all of the samples, there being more of these in mud than in water. Most of the bacteria which survived temperatures higher than 40°C. proved to be spore formers. Not many of the spore formers survived at 80°C. and too few survived boiling for ten minutes to warrant the numerical expression of an average from the available data.

TABLE 5

Number of pure cultures which multiplied after being held at the stated temperature for 10 minutes

DESCRIPTION OF CULTURES	EXPOSURE TEMPERATURE						
	20°C.	30°C.	40°C.	50°C.	60°C.	80°C.	100°C.
"C₁" from water.....	25	24	9	3	0	0	0
"C₁" from mud.............. ...	25	21	11	5	1	1	0
Stock cultures.................	78	78	36	14	8	6	2

The temperature tolerance of several pure cultures of marine bacteria was tested by noting their ability to reproduce after being heated. Nutrient sea-water broth was inoculated and distributed in serological tubes. Pairs of these were held in water baths at different temperatures for ten minutes and then cooled immediately in ice water, after which the bacteria were tested for viability. Table 5 shows the number of cultures which multiplied following this treatment.

The cultures designated "C₁", are colonies differing superficially from each other, fished directly from pre-solidified nutrient agar which had been inoculated with freshly collected samples of sea water or marine mud and incubated at 12°C. At no time were these organisms subjected to temperatures higher than 12°C. until they were tested for their temperature tolerance. The "stock" cultures, all differing either morphologically, culturally

or physiologically from each other, have been isolated over a period of years from sea water or other marine materials. They have been sub-cultured many times and maintained on seawater agar slants in the refrigerator at 0° to 4°C.

Only five cultures out of the 128 tested failed to grow after being held at 30°C. for ten minutes but many of them multiplied less rapidly, indicating that while all of the individuals comprising any one culture had not been killed, many of the individuals were injured. This was confirmed later by plate counts on the cultures and observations on the respiration of heat-treated cultures. It is noteworthy that over half of the pure cultures were killed in 10 minutes at 40°C. Similarly Bedford

TABLE 6

Oxygen consumed by suspensions of marine bacteria in two hours at 20°C. after being heated to the stated temperature for ten minutes and the number of viable bacteria in the heated suspensions

| | EXPOSURE TEMPERATURE | | | |
	20°C.	30°C.	40°C.	50°C.
Oxygen uptake (mm.³)...	0.92	0.54	0.19	0.04
Bacteria per ml. × 100,000	267	184	51	9

(1933) found that 37°C. was lethal for 40 of the 71 cultures of marine bacteria with which he was working.

According to Bronfenbrenner *et al.* (1939) the respiration of bacteria is a better criterion of their viability than their ability to reproduce in a given medium. Studies on the oxygen uptake of suspensions of marine bacteria demonstrated that many were rendered incapable of respiration by 10 minutes exposure at 30°C. The tests were made by pipetting 2.0 ml. of a heavy suspension of a 24-hour old enrichment culture of mixed marine microflora into Barcroft respirometer flasks. Duplicates of each were held in water baths at 20°, 30°, 40° and 50°C. for ten minutes, after which they were cooled immediately to 20°C. After placing 0.2 ml. of 10 per cent KOH solution in the inset the respirometer flasks were fitted to the manometers and the oxygen uptake of each suspension was noted after two hours at 20°C.

Appropriate dilutions of each suspension were plated on nutrient agar to determine the number of viable cells. The results are summarized in table 6.

It will be observed that the decrease in the number of viable bacteria as indicated by plate counts is proportional to the decrease in the oxygen uptake of the heat-treated bacteria. Similar observations were made on four different heat-sensitive pure cultures which failed to multiply after being held at 40°C. for ten minutes. The loss of the ability of heated cultures to consume oxygen indicates that the respiratory enzymes of the bacteria have been inactivated. According to Edwards and Rettger (1937) the maximum temperature tolerance of bacteria is related to the minimum temperature at which their respiratory enzymes are destroyed.

OPTIMUM TEMPERATURE OF INCUBATION

In spite of the fact that Standard Methods of Water Analysis (1933) is concerned primarily with the bacteria which are of sanitary significance and which may differ markedly from the autochthonous microflora of natural waters, many bacteriologists have taken literally the instructions to count agar plates at either 20° or 37°C. when analyzing ocean, lake or river water. Most frequently the plates have been incubated at some intermediate temperature, namely, 25° or 30°C. We have studied the effect of the temperature of incubation upon plate counts by inoculating Petri dishes in groups of 14 each with 1.0 ml. of sea water or marine mud. Duplicate plates from each sample were incubated at 4°, 12°, 18°, 22°, 25°, 30° and 37°C. After different periods of incubation the colonies were counted. Table 7 shows the average results obtained with ten samples of sea water and four of marine mud. The colony counts were calculated as percentages of the maximum colony count, assuming the latter to be the colony count on plates incubated at 18°C. for 18 days. As a matter of fact the maximum number of colonies appeared on only half of the plates incubated at 18°C. for 18 days. The maximum counts of four samples occurred on plates incubated at 12°C. and the maximum counts of the other three samples were on plates

incubated at 22°C.   Results with water and mud samples were almost the same.

For the first few days of incubation the most colonies were found on plates incubated at 25° or 30°C., but after seven to ten days the most colonies were found on plates incubated at 12° to 22°C.   The bacteria which multiply at the higher temperatures do so more rapidly and hence appear earlier as colonies than those incubated at lower temperatures.   As a rule the colonies found on the plates incubated at the higher temperatures are larger than those growing at lower temperatures and give the former

TABLE 7

Relative number of colonies appearing on nutrient agar incubated for different periods of time at different temperatures, the plate counts being expressed as percentages of the average plate count after 18 days at 18°C.

INCUBATION TIME	INCUBATION TEMPERATURE						
	4°C.	12°C.	18°C.	22°C.	25°C.	30°C.	37°C.
days	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
2	0	18	30	36	41	44	8
4	0	28	41	60	65	61	12
7	4	46	67	82	78	69	12
10	9	67	91	96	84	71	13
14	17	90	98	97	85	70	
18	26	97	100	95	82	63	
21	33	98	96	87	74	53	

plates the superficial appearance of having more colonies.   The decrease in the counts after two weeks on plates incubated at 25°C. or higher is due to the merging of colonies and the liquefaction of the agar by certain bacteria, thereby obliterating the surrounding colonies.   Very few colonies developed on the plates incubated at 37°C.

The results confirm previous experiments on the thermal sensitivity of marine bacteria and show that, in general, media inoculated with sea water or marine sediments should not be incubated at temperatures exceeding 22°C.   The greatest number of different species can be detected on plates incubated at 12°C. or lower, presumably because fewer heat-sensitive species are

inactivated and apparently all of them can grow, though slowly, at 4° to 12°C. Moreover, the lower temperatures seem to favor pigment production (Hess, 1933a) and as pointed out by ZoBell and Feltham (1934) most marine bacteria are chromogenic under favorable conditions. However, due to the slowness with which colonies form at relatively low temperatures, the maximum number of visible colonies will be found on plates incubated at 18° to 22°C. for a week or two. Except for special purposes it is not feasible to incubate plates for several weeks before counting.

DISCUSSION

Since the temperature of the ocean is monotonously constant with over 80 per cent of the water and bottom perpetually colder than 5°C., it is not surprising to find that marine bacteria are extremely thermo-sensitive. It so happens that the critical point for the majority of them is near the temperature at which nutrient agar begins to congeal. While too few bacteria are killed when the medium is properly cooled to invalidate the use of plating procedures for estimating bacterial populations, it should be emphasized that prolonged exposure at 40° to 42°C., or instantaneous exposure at temperatures a few degrees higher, is lethal for a large percentage of the bacteria from the sea and perhaps from lakes also.

Anomalously the optimum temperature for the multiplication of marine bacteria in the laboratory is several degrees higher than the environment inhabited by them. Though coming from an environment which is for the most part considerably colder than 12°C., the optimum temperature for maximum plate counts of bacteria from the sea is between 12° and 22°C. The pure cultures which have been studied have temperature optima ranging from 18° to 37°C., the range for the majority being 18° to 25°C. This has been found to be true of cultures which were isolated from plates incubated at 12°C. and which had not been subjected to higher temperatures until the tests were made. Working with 71 species of bacteria from the northern Pacific Ocean, most of which were concerned with the spoilage of fish at refrigeration temperatures, Bedford (1933) found that none of them had

optima lower than 20°C. Too many intrinsic as well as extrinsic factors are involved which influence the temperature tolerance of bacteria to speculate at this time why certain bacteria have temperature optima which are several degrees higher than the environment inhabited by them.

Bacteria which are transferred directly from their native habitat to artificial media are subjected to many abrupt environmental changes besides temperature but the inimical effect of the adverse condition will probably be proportional to the temperature in accordance with the R.G.T. or van't Hoff rule. However, if the bacteria survive the shock of transplantation and start to multiply, the resulting cultures are more tolerant of laboratory conditions in general, including temperature extremes which may be because, as expounded by Sherman and Cameron (1934), physiologically old cells withstand adverse conditions better than young ones. Consequently the temperature tolerance seems to increase and the most tolerant individuals will soon predominate in the culture as the less tolerant ones fail to multiply or perish, or as stated by Reimann (1937), "an environment unfavorable to one of two mutants will cause the elimination of one and permit the growth of the other." It may be for this reason that Kluyver and Baars·(1932) maintain that the longer a culture has been in the laboratory the less adaptive ability it possesses. Incidentally, like the experience of Casman and Rettger (1933) with members of the *"subtilis* group," our attempts to acclimate marine bacteria to temperatures higher than the maximum of 3- or 4-week old sub-cultures have been unsuccessful.

There is no evidence to indicate that temperatures as low as 0° to −5°C. injure marine bacteria although according to Hess (1933b), they slowly die at −16°C. Most of them multiply and are otherwise physiologically active until the water essential to chemical reactions is removed by solidification due to freezing. ZoBell (1934) reports that 76 out of 88 different species of marine bacteria multiplied slowly at 0° to −4°C. Bedford (1933) found that all of his 71 species except three grew at 0°C. and 23 of them grew at −5°C. Neither Bedford nor the senior author have

found true psychrophiles, or cultures which grow best at relatively low temperatures, but Hess (1933b) gives 5°C., as the optimum temperature for the multiplication of the marine bacteria which he has studied. Berry and Magoon (1934) who have been working with microorganisms which grow at sub-zero temperatures question the existence of a true cold-loving or psychrophilic flora.

SUMMARY

Many of the bacteria occurring in sea water and marine sediments are sensitive to the plating temperature of nutrient agar, there being significantly fewer which formed colonies on agar plated at 45°C. or above than on that plated at 42°C. There were only 81 to 83 per cent as many colonies which developed on nutrient gelatin plated at 45°C. as on that plated at 30°C.

Heating samples of sea water and mud to 30°C. for ten minutes killed around 25 per cent of the bacteria, and only 20 per cent of the bacteria survived 40°C. for ten minutes.

A diminution of the oxygen uptake of suspensions of heat-treated bacteria indicated that the respiratory enzymes of some forms are inactivated by temperatures as low as 30°C.

Nutrient agar inoculated with sea water or marine sediments yields maximum colony counts when incubated at 18° to 22°C. for a week or two. Very few colonies develop on plates incubated at 30° to 37°C.

Most of the bacteria isolated from the sea grow best at temperatures which are considerably higher than the marine environment inhabited by them. Although nearly all marine bacteria multiply slowly at near zero temperatures, true psychrophiles have not been found.

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CHEMOTACTIC RESPONSE TO STAPHYLOCOCCUS STRAINS OF VARYING PATHOGENICITY

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In one of Dr. Robert Chambers' recently exhibited moving pictures leucocytes were shown moving toward certain colonies of *Staphylococcus* and apparently avoiding other colonies of the same species. Dr. Chambers was good enough to agree to our making an examination.

After trying several procedures a simple cell was arranged as follows: The bacteria to be tested were grown in broth, removed by centrifuging and added to a semi-solid agar, 0.75 per cent agar in nutrient broth, held in solution at 40°C. The agar suspension of organisms was then drawn into capillary tubes with a lumen of 5 to 8 microns and the agar allowed to solidify. The agar-filled capillaries were broken into about 5 mm. lengths and one or more placed on a cover slip.

A fragment of mouse spleen, 1 to 2 cu. mm., as a source of leucocytes, was gently washed in Ringer's solution and placed on the cover slip beside the capillary tubes of bacteria. These were arranged, as shown in figure 1, so that the tips of the tubes were about 2 mm. from the spleen fragment. Where two tubes of bacteria were used, the ends of the tubes were approximately equidistant from each other and from the tissue. A drop of Ringer's solution or normal rabbit serum was then added, just large enough to cover, but not to float, the piece of tissue and to cover the capillary tubes for about half their length. The surface film of the drop held both the tissue and the tubes firmly in position. The preparations were then inverted over a depression slide and cemented in position. Strict asepsis was maintained.

The preparations were either incubated (37°) on the micro-

scope stage for continuous observation or in an ordinary incubator and examined at suitable intervals, usually after one, three and six hours. Where no bacteria were present the movement of leucocytes from all sides of the tissue fragment was more or less uniform resulting in a gradually widening peripheral zone; where a tube of chemotactically active bacteria was present there was, after twenty to thirty minutes, a distinguishable movement of leucocytes toward the end of the bacteria-containing tubes. After two to six hours, leucocytes were massed about the end of the tube. The action was at its maximum in about six hours;
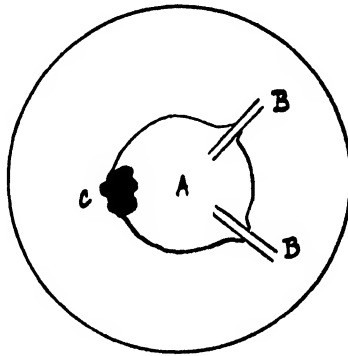
FIG. 1. HANGING DROP PREPARATION FOR OBSERVING CHEMOTACTIC ACTION OF BACTERIA ON LEUCOCYTES

A, drop of Ringer's solution; *B*, capillary tubes of bacteria in semi-solid agar; *C*, fragment of spleen.

later, bacteria grew or diffused into the fluid drop and the effect was soon destroyed. No significant difference occurred in rate of movement of leucocytes or in their directional response to the type of bacteria where the menstruum was Ringer's-gelatin solution (Rous and Turner, 1916), normal rabbit serum or various proportions of the two. In all the recorded experiments Ringer's-gelatin was used.

Where two tubes were present, each containing bacteria of similar chemotactic activity, as from the same culture, the movement toward the two tubes was always similar; where the bacteria were dissimilar in chemotactic activity, leucocytes tended

to move toward one tube and to avoid the other.   It was therefore possible to compare the relative chemotactic action of any two organisms.

Some thirteen strains of staphylococci have been examined—two hundred tests in all.   The organisms were freshly isolated strains from active lesions and normal skin, representing various grades of toxigenicity and general pathogenicity.

Each strain was compared with every other strain in from ten to thirty trials.   From the results it was possible to arrange the

TABLE 1

Relative chemotactic response of leucocytes to thirteen strains of staphylococci

ORGANISMS	NUMBER OF TRIALS	PERCENTAGE OF TRIALS IN WHICH THE TEST ORGANISM, COMPARED WITH ALL OTHERS IN THE SERIES, GIVES A CHEMOTACTIC REACTION WHICH IS:		
		Greater	Equal	Less
46 p	27	0	33	66
24 x	35	14	31	55
24 f	12	17	33	50
24 xi	11	36	27	37
46 e	5	40	0	60
46 a	14	43	36	21
24 k	26	46	27	27
125 a	26	46	31	23
24 ki	8	50	38	12
46 g	27	52	18	30
24 b	17	53	41	6
125 c	28	54	21	25
125 g	8	63	25	12

series in the order of increasing chemotactic attraction for leucocytes.   In thirty trials, in which two tubes were filled with bacteria from the same culture, the chemotactic response was the same, in so far as the method of examination permitted comparison.   On the other hand, successive trials with the same combinations of strains but using cultures made on different days did not always give the same results.   However, by making several determinations the approximate relative chemotactic action of each strain could be determined.   These results are shown in table 1.   The figures in table 1 mean, in the first case, for ex-

ample, that in twenty-seven trials of strain 46p against the other twelve strains: no other strain gave less chemotactic response; 33 per cent of trials with other strains gave an equal response; in 66 per cent of trials other strains exhibited greater chemotactic action.

It is obvious from inspection of table 1 that further tests might result in some change in the position of strains in the order of relative chemotactic action but it is equally obvious that between the two or three strains placed at the top, i.e., showing the least chemotactic action, and the two or three placed at the bottom of the list, i.e., showing the greatest chemotactic action, there is a wide difference.

The significance of arranging the strains in order of increasing chemotactic action lies in the fact that the strains at the top of the list, i.e., those showing the least chemotactic action, are the most pathogenic, yield most haemotoxin and exhibit the other characteristics which are generally present in pathogenic strains. The strains at the bottom of the list, i.e., those strains which show the greatest chemotactic action, are non-pathogenic for mice, produce little or no haemotoxin and exhibit the other characteristics of non-pathogenic strains of staphylococci. These data are summarized in table 2.

Since the least chemotactic action was shown by toxigenic strains, an attempt was made to determine the influence of toxin. A series of trials was made with strain 24x which, as indicated in table 2, produces a high yield of haemotoxin. Where duplicate tubes of bacteria from the same culture were arranged, in the manner just described, there was a uniform movement of leucocytes toward the two tubes of bacteria. This invariably occurred in trial after trial. In contrast, preparations were made as follows: Bacteria, strain 24x, were suspended in semi-solid agar and then divided into two portions. To one portion, an equal volume of toxin (haemotoxin titre 1:3000, see table 2) prepared from the same strain, was added and to the second portion of bacterial suspension an equal volume of the sterile broth, as used in the preparation of toxin, was added. These were arranged as in the previous experiments with a fragment of spleen and a tube of each of the above-mentioned mixtures. The results of one set

TABLE 2

A list of 13 strains of staphylococci arranged in order of increasing chemotactic action for leucocytes compared with pathogenicity for white mice and related characteristics

STRAIN NUMBER	CHEMOTACTIC ACTION	PATHOGENICITY*	HAEMO-TOXIN TITRE†	COLONY FORM	MUCOIDNESS	PIGMENT	MANNI-TOL FER-MENTA-TION	GELATIN LIQUEFACTION	COAGULASE	AGGLUTINATION	
										24x serum	46x serum
46 p	Least	p.	1:3000	S	Mucoid	Cream	+	24 hrs.	1 hr.	1:1000	1:1000
24 x		p.	1:3000	S	Non-mucoid	Cream	+	24 hrs.	1 hr.	1:1000	1:1000
24 f		p.	1:3000	S	Non-mucoid	Cream	+	24 hrs.	2 hrs.	1:1000	1:1000
24 xi		p.	1:3000	S	Non-mucoid	Cream	+	24 hrs.	1 hr.	1:1000	1:1000
46 e		p.	1:3000	R	Mucoid	Cream	+	24 hrs.	6 hrs.	1:1000	1:1000
46 a		Slight p	1:100	S	Non-mucoid	Cream	+	−5 da.	6 hrs.	1:100	1:80
24 k		Non p.	Nil	S	Non-mucoid	Deep cream	−	72 hrs.	0	1:1000	1:1000
125 a		Slight p	1:100	S	Non-mucoid	Deep cream	+	−5 da.	9 hrs.	1:100	1:80
24 ki		Non p.	Nil	S	Mucoid	Deep cream	−	72 hrs.	0	1:100	1:100
46 g		Non p.	Nil	S	Non-mucoid	White	−	−5 da.	0	1:1000	1:1000
24 b		Non p.	Nil	S	Non-mucoid	White	−	−5 da.	0	1:100	1:80
125 c		Non p.	Nil	S	Non-mucoid	White	+	−5 da.	0	1:1000	1:1000
125 g	Greater	Slight p	1:500	S	Non-mucoid	Cream	+	−5 da.	5 hrs.	1:1000	1:1000

* Pathogenicity was tested by intraperitoneal injection of young broth cultures into white mice: p = small doses killed, slight p = only very large doses were effective, non p = very large doses produced no effect.

† The figures indicate the dilution of toxin, prepared in semi-solid agar (Burnet, F. M., 1930, Jour. Path. Bact., **33**, 1 Dolman, C. E., 1932, Can. Pub. Health Jour., **23**, 125) which haemolize a standard suspension of rabbit red cells.

of trials are summarized in table 3, showing clearly that the addition of toxin appreciably decreases the chemotactic influence of these organisms on leucocytes. Similar results were obtained with other high toxigenic strains.

In interpreting these results it should be noted that since this is a toxigenic strain, toxin is probably present in all the preparations or is produced during the six hours' incubation. The difference therefore between the two preparations is in the amount of toxin present, rather than in its presence or absence.

As a further indication that the decreased chemotactic action results from toxin, filtrates from cultures of non-toxigenic strains like 24k, prepared in the same manner as toxin, when added to bacterial suspensions had no influence on leucocyte movement.

TABLE 3

Movement of leucocytes toward bacteria compared with bacteria from the same culture + added toxin

LEUCOCYTES SHOW:	NUMBER OF TRIALS
Equal movement toward bacteria only and bacteria + toxin	8
Greater movement toward bacteria than toward bacteria + toxin	16
Less movement toward bacteria than toward bacteria + toxin.	0

Whether the haemotoxin itself produces a negative chemotactic or repellant action on leucocytes, or whether some other substance, such as leucocidin, is produced by the bacteria which produce haemotoxin is not apparent at present. Our efforts to assay the preparations for leucocyte-destroying substances by the various methods of methylene-blue reduction and direct observation of leucocyte disintegration have been too variable to have any quantitative significance.

An attempt was made to measure the chemotactic action of these strains *in vivo*. The organisms from 18-hour broth cultures were removed by centrifuging and resuspended in Ringer's solution to 10 organisms per milliliter and 0.5 amounts injected intraperitoneally into mice. Six hours later the mice were killed, the peritoneal cavity carefully washed with 10 ml. of Ringer's solution and haemocytometer counts of white cells made. On the average, approximately 50 per cent higher counts were

obtained on the peritoneal washings of mice injected with strains of staphylococci which gave high chemotactic responses in the hanging drop preparations than on the peritoneal washings of mice injected with strains of staphylococci showing low chemotactic response *in vitro*.   However, the variation in counts in different mice injected with similar preparations was so great that the results are of doubtful significance.

Since this work was completed it was shown by Rigdon (1939) that when non-toxin-producing strains of staphylococcus are injected into mice, leucocytes rapidly invade the area of infection followed by phagocytosis of the bacteria.   When toxin-forming staphylococci are injected, while the initial leucocytic response is similar to that following the injection of non-toxin-forming bacteria, later leucocytes were abundant in areas where there were no bacteria but only slightly infiltrated areas rich in bacteria.   In these areas, moreover, phagocytosis was not at any time a conspicuous process.   Rigdon suggests that the difference results from the destruction of leucocytes by toxin.   It seems apparent from our results that negative chemotactic action of toxin may be an equally important factor.   Indeed, Pettersson (1939) suggests that antibodies which neutralize negative chemotactic substances produced by bacteria constitute an important factor in immunity.

SUMMARY

It has been shown that strains of staphylococci of low pathogenicity which produce little or no haemotoxin exhibit relatively more chemotactic action for leucocytes than strains of high pathogenicity and high toxigenic properties.   Evidence has been advanced to suggest that a negative chemotactic action of toxin accounts for this difference.

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A PHOTOMICROGRAPHIC STUDY OF THE RATE OF GROWTH OF SOME YEASTS AND BACTERIA

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In a series of papers, Bayne-Jones and Adolph (1932a and b) and Adolph and Bayne-Jones (1932) published their analyses of cinematographic records of growing yeasts and bacteria. Of special interest is the course of the growth *in substance* of *Saccharomyces cerevisiae*, which proceeds in waves. The authors have shown that this is the result of a much reduced growth rate during and before budding. With bacteria, no such waves were observed in the growth curve. It seemed therefore, worth while to ascertain whether a wavy growth curve is encountered only in budding organisms or whether it is typical of yeasts, as compared with bacteria, regardless of their method of vegetative multiplication.

EXPERIMENTAL

The method used in the present investigation is photography in dark field. The dividing yeasts or bacteria were grown on the surface of a thin agar layer under cover glass, and serial photographs taken with a 20-amps. carbon arc as a source of light. A spherical glass flask filled with water was used, both as a condensing lens and as a heat filter. Stray light and heat were shut off from the microscope by a thick asbestos board with a circular hole in its center to accommodate the condensing flask. A thermometer suspended over the stage and parallel to the tube of the microscope indicated the temperature which was recorded before and after each exposure. The temperature usually remained within 1 to 1.5°C.

The agar film was prepared as follows: Two rectangular cover glass strips are immersed in melted paraffine in a Petri dish, taken out one at a time with forceps, allowed to drain and placed apart and parallel to one another on a glass slide. The long edges of the strips should be perpendicular to the long edge of the slide. The distance between the inner edges of the strips should be slightly less than the length of the cover glass which they are intended to support. The slide, with the glass strips in position, are now passed over a flame, which would sterilize the system and melt the paraffine, and deposited in a Petri plate. Upon cooling, the paraffine cements the glass strips to the slide. Now, a loopful of a paper-filtered agar medium is deposited on the slide between the glass strips. This assumes the shape of a plano-convex lens with the spherical surface slightly above the level of the glass strips. The surface of the agar is inoculated very lightly with yeasts or bacteria and a sterile cover glass is placed on the surface of the agar and pressed down until it rests on the glass strips. This, of course, flattens the agar, often without breaking it, and gives a preparation optically suitable for observation in dark field. The preparation is now sealed with vaspar to prevent it from drying, and the sealed chamber contains sufficient air for the need of aerobic bacteria.

The organisms investigated were *Schizosaccharomyces pombe* and *Bacillus cereus*. In calculating volumes, the dark field picture of the first was considered to approach more nearly an ellipsoidal form, while the form of the second was considered to be cylindrical.

RESULTS

Growth and reproduction curves of *Schizosaccharomyces pombe* are plotted in the graph of figure 1. In addition to total growth in volume (curve V) and in length (curve L), a curve illustrating the growth, in length, of a single cell is also given (curve I). Due to the relative constancy of the width, changes in cell length are proportional to changes in volume. This is indicated by the general parallelism between curves V and L except at the very beginning where an apparent anomaly is due

to the fact that the resting ancestor-cell with which we started the reported experiment decreased slightly in thickness before it assumed the morphology of growing cells. The slight decrease in volume observed may be due to the initial feeding of the
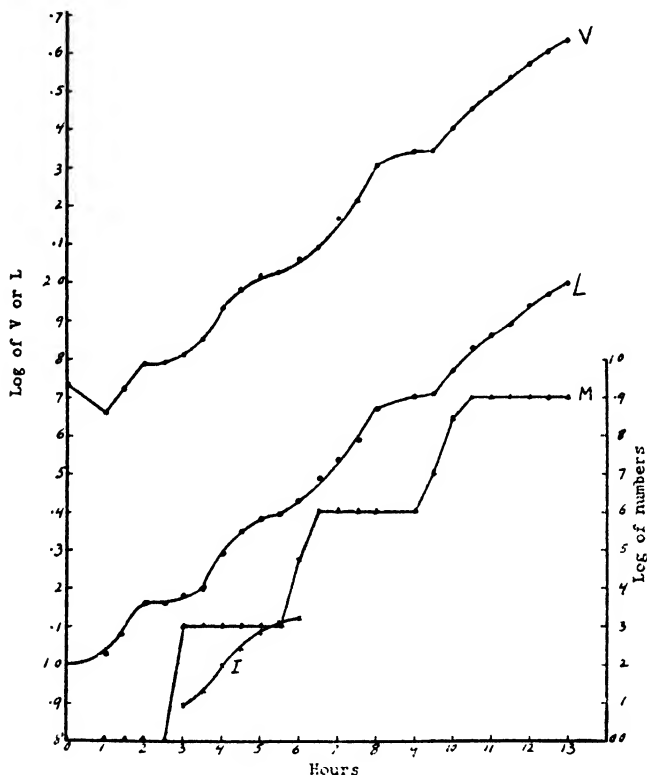
FIG. 1. GROWTH AND REPRODUCTION CURVES FOR SCHIZOSACCHAROMYCES POMBE
V = logarithm of the total volume in μ^3 against time in hours; L = logarithm of the total length in μ against time in hours; M = multiplication curve plotted as logarithm of the number of cells against time in hours; I = logarithm of the length in μ against hours for cell IA.

resting cell on its own reserve material. These curves indicate clearly that growth, *in substance*, of the dividing yeast ceases, or practically ceases, at the time of cell division, to be resumed afterwards. An anomaly is presented by cell IAA, which goes through two maxima of growth rate during its life span. We are at present unable to offer a satisfactory explanation of this be-

havior. As the number of cells increases, and successive generations get out of phase with each other, the resultant effect on the curve of total growth is a gradual smoothing of its wavy course.

TABLE 1

Total and individual average rates of growth in length of Schizosaccharomyces pombé in millimicrons per micron of living yeast per minute, at 28 to 29°C. in consecutive intervals of 30 minutes each

MATERIAL	INTERVAL	AVERAGE RATE PER MINUTE	MATERIAL	INTERVAL	AVERAGE RATE PER MINUTE
		mμ			*mμ*
1. Total growth...	1		3. Cell IAA......	1	3.3
	2			2	6.2
	3	4.5		3	2.3
	4	5.8		4	6.5
	5	0.0		5	0.8*
	6	1.7		6	0.6*
	7	1.4	4. Cell IAB	1	0.6
	8	8.4		2	3.0
	9	4.2		3	5.2
	10	3.2		4	6.8
	11	0.6		5	1.3*
	12	3.4		6	0.9*
	13	4.4		7	0.9
	14	3.9	5. Cell IB	1	3.6
	15	4.1		2	5.3
	16	7.5		3	3.8
	17	1.3*		4	3.3
	18	0.8*		5	2.0
	19	0.7		6	0.9
	20	5.1	6. Cell IBA	1	3.9
	21	4.8		2	3.7
	22	2.3		3	4.1
	23	2.6		4	1.0*
	24	3.7		5	1.1*
	25	2.7	7. Cell IBB......	1	3.2
	26	2.2		2	9.9
2. Cell IA.........	1	3.0		3	4.7
	2	5.6		4	1.2*
	3	3.5		5	0.2*
	4	2.8			
	5	1.7			

* Value obtained by extrapolation.

In table 1 we have calculated the average rate of growth in length within consecutive 30-minute intervals, which we expressed in terms of rate of increase in length of one micron of living yeast per minute.

Similar experiments with *Bacillus cereus* are illustrated in figure 2, and the average rate of growth in length in each con-
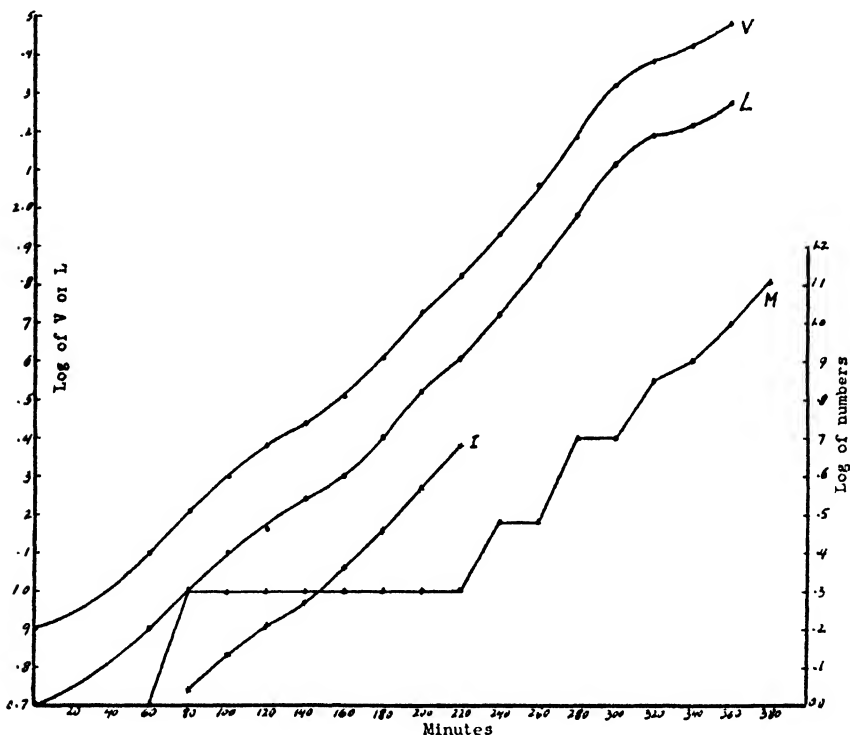
FIG. 2. GROWTH AND REPRODUCTION CURVES FOR BACILLUS CEREUS

The letters have the same meaning as in Figure 1; the unit of time here is, however, the minute instead of the hour as in figure 1.

secutive 20-minute interval is given in table 2. They indicate that in the case of *Bacillus cereus*, the curves of growth *in substance* are gently wavy, although no clear correlation is found between cell division and change in the rate of growth. This study furthermore indicates that the cell division of actively growing bacteria is not a regular process. Perhaps the change

TABLE 2

Total and individual average rates of growth in length of Bacillus cereus in
millimicrons per micron of living bacteria per minute, at 31 to 32.5°C.
in consecutive intervals of 20 minutes each

MATERIAL	INTERVAL	AVERAGE RATE PER MINUTE	MATERIAL	INTERVAL	AVERAGE RATE PER MINUTE
		$m\mu$			$m\mu$
	1	4.3*		1	12.4
	2	8.1*		2	9.5
	3	12.17*		3	7.5
	4	14.1	2. Cell IA.	4	11.6
	5	12.1		5	13.2
	6	8.2		6	14.6
	7	9.0		7	14.0
	8	8.5	3. Cell IAA.......	1	18.5
	9	12.2	4. Cell IAB.......	1	40.3
1. Total growth..	10	15.8		1	10.6
	11	12.0		2	9.3
	12	14.0		3	7.4
	13	17.8		4	8.2
	14	16.6	5. Cell IB......	5	9.9
	15	17.5		6	15.8
	16	10.9		7	10.7
	17	1.8		8	14.4
	18	7.9	6. Cell IBA.	1	15.7
	19	7.6		1	14.7
			7. Cell IBB . . .	2	6.6
				3	5.6

* Value obtained by extrapolation.

in the growth rate during the life span of a single bacterial cell corresponds to some biological process other than cell division.

SUMMARY

The rate of growth *in substance* of the cell of *Schizosaccharomyces pombe* becomes *nil* or nearly so at the time of cell division, a phenomenon already known in budding yeasts. The cells of *Bacillus cereus* show no such correlation in an actively growing culture, although the rate of growth may fluctuate within the life span of the cell.

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INHIBITING EFFECT OF ACETIC ACID UPON MICRO-ORGANISMS IN THE PRESENCE OF SODIUM CHLORIDE AND SUCROSE[1,2]

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Vinegar has long been added to foods in the home and in the commercial packing plant as a flavoring element; in addition, it has a definite preservative action against spoilage microorganisms. Any toxic effect of vinegar is undoubtedly due to its acetic acid content.

Levine and Fellers (1940) showed that the toxicity of acetic acid is not due to pH alone although an increase in the hydrogen ions resulted in a decrease in heat resistance of the bacteria studied.

As vinegar is usually associated with sugar or salt or both in a food, it seemed advisable to study the toxic effect of acetic acid in the presence of these substances.

LITERATURE REVIEW

The uses of vinegar in foods are legion: mayonnaise dressings, prepared mustard, horse-radish, pickles, salads, marinated fish, spinach, and beets are only a few of the products with which it is used. Rowse (1928) attributed the keeping qualities of mayonnaise to its vinegar content and pointed out that the preserving power of vinegar varies directly with its acetic acid content. Pederson and Breed (1926) found that one per cent acetic acid

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[1] Contribution 361, Massachusetts Agricultural Experiment Station, Amherst. Taken from Doctor's Dissertation of A. S. Levine (1939), Massachusetts State College.

[2] Presented at the Forty-First General Meeting of the Society of American Bacteriologists, New Haven, Conn. December 29, 1939. J. Bact. **39**, 17.

stopped the growth in tomato catsup of all microorganisms studied and that combinations of salt and sugar with the acid did not lower appreciably the amount of acetic acid required.

The antiseptic action of acetic acid and some other organic acids is not confined to bacteria; these acids appear to be toxic to yeasts and molds as well. Cruess and Hascal (1924) reported that 0.8 to 1.0 per cent acetic acid seemed to inhibit the fermentation of apple cider by Burgundy yeast. Katagiri (1926) found that at a constant concentration of free acid, the rate of fermentation by yeast was independent of the concentration of either acetate or formate salts. Similarly, the nature of the salt cation, Na, K, or NH_4 had no influence. The yeast fermentation was, however, very sensitive to the presence of free acid. This investigator states that the effect is no doubt a complex one, due in part to hydrogen ions and in part to the undissociated molecules of the acid but that the influence of the latter is much greater than that of the former.

Fabian and Wadsworth (1939) demonstrated that acetic acid is a better preserving agent for sweet pickles than lactic acid. The pH value of the pickle syrup was not a reliable indicator of the preserving power of the acids present. Levine and Fellers (1940) conducted comparative studies and showed that acetic acid was more toxic than either lactic or hydrochloric acid to *Salmonella aertrycke, Saccharomyces cereviseae,* and *Aspergillus niger.* These organisms were inhibited or destroyed at a higher pH value when acetic acid was used as the toxic agent than where lactic or hydrochloric acids were used.

EXPERIMENTAL

Index organisms

The test microorganisms used were *Salmonella aertrycke, Staphylococcus aureus, Phytomonas phaseoli, Bacillus cereus; Saccharomyces cereviseae,* (Lister strain) and *Aspergillus niger.* These microorganisms were selected, not only for their association with food spoilage, but also for their representative value as more or less typical members of certain microbial groups.

Methods

The effect of acetic acid on the total destruction and on the reduction of numbers of bacteria and yeast was determined by a 15-minute contact with acetic acid solutions. The test micro-organisms were also subjected to contact with acetic acid solutions containing 5 per cent salt and with acidified 20 per cent sugar syrups. The solutions were prepared as follows: Five milliliters of sterile distilled water were added by pipette to each of a series of sterile tubes. To the first tube, were added 5 ml. of acetic acid of known concentration. After mixing, 5 ml. of this solution were transferred to the next tube. The procedure was repeated until each tube in the series contained 5 ml. of acid solution in decreasing order of concentration, 5 ml. from the last tube having been discarded. One-tenth ml. of a diluted broth culture was added to each tube. The tubes were well agitated and replaced in the rack for 15 minutes. After this contact period a standard quantitative plating was made on each tube. Nutrient agar was used for the bacteria and glucose agar for the yeast.

The same general procedure was followed where acid-sugar syrups and acid-salt brines were used. Five milliliters of sterile double strength salt brine or sugar syrup were added to the first tube so that dilution with an equal volume of acetic acid gave the same strength brine or syrup in this tube as in the others of the series.

Immediately after plating, determination of pH was made on each solution remaining in the tubes. All pH determinations were made electrometrically by means of a Beckman glass electrode pH meter in which the electrode was balanced against a standard cell. Total acidities were calculated, since the acid concentration of the first tube and the subsequent dilutions were known. These calculations were checked occasionally by actual titration with standardized sodium hydroxide.

The mold was cultivated in flasks of 0.5 per cent glucose broth alone and also in glucose broth with 5 per cent salt and with 20 per cent sugar. The broth solutions in each series were acidified with acetic acid. An unacidified control was used in each series also.

The technique employed in the study of *Aspergillus niger* was somewhat different from that used with bacteria and yeast due to the nature of the mold. Sterile 50 ml. portions of 0.5 per cent glucose broth were transferred aseptically to sterile 250 ml. Erlenmeyer flasks. This procedure was used to obviate unequal loss of flask contents through evaporation during sterilization. The sterile sugar- and salt-glucose broths were added to series of flasks in the same way. Varying amounts of acetic acid were added to the flasks to make series of decreasing acid concentrations. The volume of acid added was sufficiently small to affect the surface of the medium but little. Ten milliliters of solution were removed from each flask for pH and total acidity determinations. Acidity was determined by titration with sodium hydroxide with phenolphthalein as the indicator. The remaining 40 ml. gave a maximum surface in each flask for mold growth. A 0.1 ml. water suspension of spores of *Aspergillus niger* was inoculated into each flask. The flasks were held for five days at 30°C. (80°F.) in the absence of light. The dried weight of mold which had developed during the incubation period was determined by filtering and washing the flask contents. The filtrate was saved for pH and total acidity determinations. The mold mats were dried at 100°C. (212°F.) to a constant weight.

Experimental results

Results are presented in tables 1 to 6. The percentage reduction in whole numbers is based on the number of organisms surviving after a 15-minute contact period with similar solution, water, brine, or syrup, that contained no added acetic acid. Thus, in table 1 at a pH 3.3 and a total acidity of 0.15 per cent acetic acid, there was a 98 per cent reduction in the number of cells of *Salmonella aertrycke* surviving from the number obtained when water at pH 6.2 was used as the contact medium. For brine of the same total acidity, 0.15 per cent, the pH was 3.1 and the reduction was 100 per cent. A 20 per cent sucrose syrup that contained 0.15 per cent acetic acid and had a pH of 3.5 caused a reduction from the number of organisms surviving in the unacidified syrup of 99 per cent. Greater numbers of *Salmonella aer-*

trycke survived contact with the unacidified 5 per cent brine and the unacidified 20 per cent syrup than with the water alone.   Bushnell (1921) found that broth media containing 4 per cent NaCl

TABLE 1

Effect of different concentrations of acetic acid on the survival of Salmonella aertrycke in water, salt brine, and sugar solutions

Contact period, 15 minutes

TYPE OF SOLUTION	ACIDITY OF SOLUTION		TOTAL COUNT	REDUCTION IN NUMBERS
	pH	Calculated total acidity		
		per cent	*bacteria per ml.*	*per cent*
Water + acetic acid........	3.3	0.15	1,870	98
Water + acetic acid	3.4	0.08	4,800	94
Water + acetic acid..	3.6	0.04	8,400	90
Water + acetic acid....	3.8	0.02	13,800	84
Water + acetic acid	4.0	0.01	24,000	71
Water + acetic acid....	4.2	0.005	36,000	57
Water only	6.2	0.0	84,000	
5 per cent brine + acetic acid....	3.1	0.15	0	100
5 per cent brine + acetic acid.	3.3	0.08	2	99+
5 per cent brine + acetic acid	3.5	0.04	6,000	94
5 per cent brine + acetic acid. ..	3.7	0.02	14,400	85
5 per cent brine + acetic acid	4.0	0.01	48,000	50
5 per cent brine + acetic acid..	4.3	0.005	84,000	14
5 per cent brine only.	6.1	0.0	96,000	
20 per cent syrup + acetic acid . .	3.5	0.15	1,320	99
20 per cent syrup + acetic acid .	3.7	0.08	7,200	94
20 per cent syrup + acetic acid	4.0	0.04	15,600	87
20 per cent syrup + acetic acid.........	4.4	0.02	21,600	82
20 per cent syrup + acetic acid.. . .	4.7	0.01	72,000	40
20 per cent syrup + acetic acid	5.3	0.005	108,000	10
20 per cent syrup only.	7.1	0.0	120,000	

192,000 bacteria were added to each ml. of the solution.

were more favorable for growth of the organisms studied than broth containing one per cent.   At the higher total acid concentrations, the brine solution was slightly more effective than the water or the syrup solutions in reducing the number of cells of *Salmonella aertrycke.*   However, at the lower acid concentrations

the brine seemed to be less effective than the water of corresponding total acidity, in reducing the number of organisms. The

TABLE 2

Effect of different concentrations of acetic acid on the survival of Staphylococcus aureus in water, salt brine, and sugar solutions

Contact period, 15 minutes

TYPE OF SOLUTION	ACIDITY OF SOLUTION		TOTAL COUNT	REDUCTION IN NUMBERS
	pH	Calculated total acidity		
		per cent	*bacteria per ml.*	*per cent*
Water + acetic acid..	3.3	0.17	17	99+
Water + acetic acid	3.4	0.08	114	99
Water + acetic acid....................	3.6	0.04	260	97
Water + acetic acid	3.8	0.02	2,400	76
Water + acetic acid.	4.0	0.01	5,000	50
Water + acetic acid	4.2	0.005	9,200	8
Water + acetic acid.	4.4	0.003	10,800	
Water only.	6.2	0.0	10,000	
5 per cent brine + acetic acid..........	3.1	0.17	0	100
5 per cent brine + acetic acid..........	3.2	0.08	14	99+
5 per cent brine + acetic acid	3.4	0.04	315	96
5 per cent brine + acetic acid..........	3.6	0.02	1,200	83
5 per cent brine + acetic acid..........	3.7	0.01	1,500	79
5 per cent brine + acetic acid..........	4.0	0.005	4,200	42
5 per cent brine + acetic acid..........	4.2	0.003	6,400	11
5 per cent brine only.......	6.1	0.0	7,200	
20 per cent syrup + acetic acid........	3.5	0.17	0	100
20 per cent syrup + acetic acid.........	3.7	0.08	1	99+
20 per cent syrup + acetic acid........	4.0	0.04	3	99+
20 per cent syrup + acetic acid.........	4.3	0.02	61	99
20 per cent syrup + acetic acid.........	4.7	0.01	900	79
20 per cent syrup + acetic acid.........	5.1	0.005	3,600	14
20 per cent syrup + acetic acid.	5.9	0.003	6,000	
20 per cent syrup only	7.1	0.0	4,200	

7,200 bacteria were added to each ml. of the solution.

brine was also less effective than the syrup at equivalent pH but this is not surprising inasmuch as the syrup contained a slightly higher percentage of the acetic acid.

Table 2 shows that plain water reduced the numbers of *Staphy-*

lococcus aureus less than did the unacidified salt or sugar solutions. At the high concentrations of acid, all three solutions were about equally effective in reducing the numbers of added viable bacteria. At pH 4.2 the percentage reduction was approximately the same in the water and in the 5 per cent salt brine solutions, being 8 and 11 per cent, respectively. However, at pH 4.3 in the 20 per cent sugar syrup series, the number of living cells was reduced 99 per cent. In this case some of the reduction may be attributed to the slightly higher acid concentration at this pH value. The syrup permitted the survival of no organisms at pH 3.5 and acidity of 0.17 per cent, whereas at pH 3.3 and the same total acidity, the water solution permitted the survival of some organisms.

Table 3 presents the results obtained on an experiment of a similar nature with *Phytomonas phaseoli*, a plant pathogen. This organism proved to be less resistant to acetic acid than did the other microorganisms. Water and acetic acid at pH 3.4, and brine and syrup at pH 4.0 caused a 100 per cent destruction of the organisms in 15 minutes. In addition, both the brine and the syrup controls with no added acid were more toxic than the water alone. Again, the acidified sugar syrup at any pH caused a greater reduction in numbers than did the brine or water series solution of a corresponding hydrogen-ion concentration. This was true, despite the fact that the unacidified sugar syrup was less toxic to *Phytomonas phaseoli* than the plain salt brine.

Bacillus cereus, an organism closely related to *Bacillus mesentericus* which causes a "ropy" spoilage in many foods, yielded the results in table 4. The unacidified 20 per cent sugar solution seemed to enhance the survival of *Bacillus cereus*, whether as vegetative cells or as spores. But, the addition of acid to the syrup again caused a greater reduction in numbers of surviving organisms than that obtained after contact with brine or water solutions of a corresponding pH value. Although the brine alone was slightly more toxic than the water alone, the latter, when acidified with acetic acid, caused a greater reduction in numbers than the former at the same total acid concentration. If one compares the results on corresponding hydrogen-ion concentration, the difference becomes more marked due, probably, to the

higher acid concentration in the water solution. The same trend, in general, is observed in the case of spores although there are a

TABLE 3

Effect of different concentrations of acetic acid on the survival of Phytomonas phaseoli in water, salt brine, and sugar solutions

Contact period, 15 minutes

TYPE OF SOLUTION	ACIDITY OF SOLUTION		TOTAL COUNT	REDUCTION IN NUMBERS
	pH	Calculated total acidity		
		per cent	*bacteria per ml.*	*per cent*
Water + acetic acid	3.3	0.15	0	100
Water + acetic acid	3.4	0.08	0	100
Water + acetic acid....................	3.6	0.04	41	99+
Water + acetic acid.	3.8	0.02	69	99
Water + acetic acid...	4.0	0.01	167	98
Water + acetic acid.....	4.2	0.005	5,000	50
Water + acetic acid.	4.4	0.002	6,000	40
Water only....................	6.2	0.0	10,000	
5 per cent brine + acetic acid..........	3.1	0.15	0	100
5 per cent brine + acetic acid.....	3.3	0.08	0	100
5 per cent brine + acetic acid..........	3.5	0.04	0	100
5 per cent brine + acetic acid..........	3.7	0.02	0	100
5 per cent brine + acetic acid..........	4.0	0.01	0	100
5 per cent brine + acetic acid.........	4.3	0.005	30	98
5 per cent brine + acetic acid.	4.6	0.002	440	68
5 per cent brine only.	6.1	0.0	1,380	
20 per cent syrup + acetic acid	3.5	0.15	0	100
20 per cent syrup + acetic acid	3.7	0.08	0	100
20 per cent syrup + acetic acid.........	4.0	0.04	0	100
20 per cent syrup + acetic acid.	4.4	0.02	24	99+
20 per cent syrup + acetic acid	4.7	0.01	900	86
20 per cent syrup + acetic acid.	5.3	0.005	4,200	36
20 per cent syrup + acetic acid	6.5	0.002	5,400	18
20 per cent syrup only.............	7.1	0.0	6,600	

7,200 bacteria were added to each ml. of the solution.

smaller number of organisms involved. Since acetic acid appears to be as effective against spores as against vegetative cells, it is significant that it may be useful for the control of spore-forming bacteria in foods.

Yeasts can generally tolerate higher acidities than most bacteria. Table 5 shows that *Saccharomyces cereviseae* survived contact with acidified water, brine, and syrup solutions at higher total

TABLE 4

Effect of different concentrations of acetic acid on the survival of Bacillus cereus in water, salt brine, and sugar solutions

Contact period, 15 minutes

TYPE OF SOLUTION	ACIDITY OF SOLUTION		TOTAL COUNT; BACTERIA + SPORES	REDUCTION IN NUMBERS	TOTAL COUNT; SPORES ONLY	REDUCTION IN NUMBERS
	pH	Calculated total acidity				
		per cent	per ml.	per cent	per ml.	per cent
Water + acetic acid............	3.3	0.17	68	79	5	93
Water + acetic acid............	3.4	0.08	85	74	17	76
Water + acetic acid. .	3.6	0.04	94	71	16	77
Water + acetic acid.	3.8	0.02	99	70	17	76
Water + acetic acid....	4.0	0.01	125	62	21	70
Water + acetic acid...	4.2	0.005	150	55	27	61
Water only.........	6.2	0.0	330		70	
5 per cent brine + acetic acid	3.1	0.17	80	68	4	93
5 per cent brine + acetic acid ...	3.2	0.08	95	62	13	78
5 per cent brine + acetic acid	3.4	0.04	93	63	15	75
5 per cent brine + acetic acid.... ..	3.6	0.02	101	60	16	73
5 per cent brine + acetic acid......	3.7	0.01	140	44	25	58
5 per cent brine + acetic acid.... .	4.0	0.005	190	24	26	57
5 per cent brine only...	6.1	0.0	250		60	
20 per cent syrup + acetic acid......	3.5	0.17	95	87	10	87
20 per cent syrup + acetic acid....	3.7	0.08	108	85	17	78
20 per cent syrup + acetic acid... .	4.0	0.04	114	84	20	75
20 per cent syrup + acetic acid	4.3	0.02	126	83	20	75
20 per cent syrup + acetic acid . .	4.7	0.01	150	79	22	72
20 per cent syrup + acetic acid.....	5.1	0.005	210	71	25	68
20 per cent syrup only............ .	7.1	0.0	720		70	

360 bacteria and spores were added to each ml. of the solution.
84 spores were added to each ml. of the solution.

acidities and greater hydrogen-ion concentrations than any of the bacteria studied. The acidity range of the solutions was from 0.0 to 4.73 per cent. A comparison of the water series with the brine series again shows that toxicity is due in part to the acetic

acid itself, without the influence of hydrogen-ion concentration. Although the plain brine was slightly more toxic than the water, the acidified brine caused a smaller reduction in surviving cells

TABLE 5

Effect of different concentrations of acetic acid on the survival of Saccharomyces cereviseae (Lister strain) in water, salt brine, and sugar solutions

Contact period, 15 minutes

TYPE OF SOLUTION	ACIDITY OF SOLUTION		TOTAL COUNT	REDUCTION IN NUMBERS
	pH	Calculated total acidity		
		per cent	*yeast cells per ml.*	*per cent*
Water + acetic acid....................	2.5	4.73	0	100
Water + acetic acid....................	2.7	2.37	78	88
Water + acetic acid....................	2.8	1.18	300	55
Water + acetic acid....................	3.0	0.59	420	37
Water + acetic acid....................	3.1	0.30	470	30
Water + acetic acid....................	3.3	0.15	500	25
Water only...........................	6.2	0.0	670	
5 per cent brine + acetic acid....	2.2	4.73	0	100
5 per cent brine + acetic acid.....	2.4	2.37	20	97
5 per cent brine + acetic acid..........	2.6	1.18	370	38
5 per cent brine + acetic acid..........	2.7	0.59	440	27
5 per cent brine + acetic acid.	2.9	0.30	410	32
5 per cent brine + acetic acid..........	3.0	0.15	450	25
5 per cent brine only..................	6.1	0.0	600	
20 per cent syrup + acetic acid.........	2.5	4.73	0	100
20 per cent syrup + acetic acid...... ..	2.7	2.37	15	97
20 per cent syrup + acetic acid...	2.9	1.18	280	44
20 per cent syrup + acetic acid.........	3.1	0.59	390	22
20 per cent syrup + acetic acid.........	3.3	0.30	470	6
20 per cent syrup + acetic acid.........	3.6	0.15	490	2
20 per cent syrup only.................	7.1	0.0	500	

900 yeast cells were added to each ml. of the solution.

than did the acidified water at a corresponding pH. Thus, at pH 3.0 the brine caused a reduction of 25 per cent whereas a 15-minute contact period with water at this pH resulted in a reduction of 37 per cent. Yet the water solution at pH 3.0 had a

total acidity of 0.59 per cent while the brine at the same pH had only 0.15 per cent acidity. The greater toxicity of the water solution seems to be due, therefore, to the higher content of acetic

TABLE 6

Effect of different concentrations of acetic acid on the growth of Aspergillus niger in flasks containing glucose broth, glucose broth brine, and glucose broth syrup

MEDIUM	INITIAL ACIDITY		FINAL ACIDITY		DRIED MOLD WEIGHT
	pH	Titratable acidity	pH	Titratable acidity	
		per cent		per cent	mgm.
Glucose broth................................	4.1	0.27	4.1	0.27	0
Glucose broth.........................	4.3	0.21	4.3	0.21	17
Glucose broth................................	4.4	0.16	7.1	0.03*	162
Glucose broth...........	4.5	0.15	7.6	0.03	148
Glucose broth..........................	4.7	0.10	7.6	0.03	133
Glucose broth..........................	5.2	0.07	7.6	0.03	121
Glucose broth (control).....................	6.8	0.03	7.6	0.03	128
Glucose broth + 5 per cent NaCl....	4.0	0.27	3.9	0.32	0
Glucose broth + 5 per cent NaCl.......... .	4.1	0.22	4.2	0.18	33
Glucose broth + 5 per cent NaCl............	4.2	0.17	7.5	0.03*	132
Glucose broth + 5 per cent NaCl............	4.5	0.12	7.5	0.03	127
Glucose broth + 5 per cent NaCl.....	4.6	0.10	7.6	0.03	129
Glucose broth + 5 per cent NaCl (control)..	6.7	0.03	7.6	0.03	131
Glucose broth + 20 per cent sucrose.	4.1	0.27	4.1	0.27	0
Glucose broth + 20 per cent sucrose.......	4.2	0.22	4.2	0.20	6
Glucose broth + 20 per cent sucrose.........	4.3	0.20	3.2	0.49	64
Glucose broth + 20 per cent sucrose.........	4.4	0.16	2.2	2.47	463
Glucose broth + 20 per cent sucrose.........	4.7	0.11	2.3	2.36	327
Glucose broth + 20 per cent sucrose (control).	6.8	0.03	2.1	3.02	223

The inoculation consisted of 0.1 ml. of a water suspension of *Aspergillus niger* which contained 2000 spores.

* At final pH values of more than 7.0 the apparent acidity represents a blank which is not subtracted in this table.

acid as it could not be due to any difference in the concentration of hydrogen-ions. Bach (1932) stated that generally the hydrogen-ions control antiseptic effect but that the undissociated part of lactic acid is the active factor when pH is such as to be unimportant.

The data in table 6 show the effect of different concentrations of acetic acid on the growth of *Aspergillus niger* in glucose broth, glucose broth with 5 per cent sodium chloride salt, and glucose broth with 20 per cent sucrose. The brine control and the glucose broth control yielded about the same amount of dried mold, 131 and 128 mgm., respectively. However, growth in glucose broth control containing 20 per cent sucrose yielded a dried mold mat weighing 223 grams. This mold growth was sufficient to form 3.02 per cent acid from the sucrose and increase the hydrogen-ion concentration to pH 2.1. The acid produced is calculated as acetic acid for purposes of comparison, although *Aspergillus niger* readily produces citric, oxalic, and gluconic acids when grown in sugar solution. Henrici (1930) states that on prolonged incubation the molds slowly utilize the acids they form thus causing the reaction to return toward neutrality. This is probably what occurred in the acidified broth where sugar was not present and which had mold growth. The acidities were completely utilized from the start and the reaction went beyond the neutral point. In all three series, 0.27 per cent acetic acid, or more, was necessary to prevent the growth of mold. Also, inhibition was about the same in all flasks containing 0.21 and 0.22 per cent acid as 17, 6, and 33 mgm. may be considered as equal insofar as dried mold weight is concerned (see fig. 1). Kirby, Frey, and Atkin (1935) found that the growth of *Aspergillus niger* was inhibited in a bread medium set at pH 3.5 and which contained 0.2 per cent acetic acid.

The heaviest mold mat obtained (see table 6), developed in the 0.5 per cent glucose broth containing 20 per cent sucrose and 0.16 per cent acid with a pH of 4.4. The dried mold weighed 463 mgm. A titratable acidity of 2.47 per cent with pH 2.2 was produced in the medium. In general, the sugar-broth series promoted greater mold development than did either of the other two series at corresponding acidities. The mold growths in the glucose brine series and in the plain glucose series were about the same. At a total acidity of 0.17 per cent, the former yielded 132 mgm. of dried mold whereas the latter at a total acidity of

0.16 per cent yielded 162 mgm. Yet the salted broth had a
pH 4.2 which was lower than the pH 4.4 of the 0.16 per cent plain
broth solution. This might have inhibited initial growth of the
mold sufficiently to account for the difference, as a pH value of
4.2 is close to the inhibiting limit of acetic acid for *Aspergillus
niger*. At pH 4.5 the plain glucose broth had an acidity of 0.15

FIG. 1 Growth of *Aspergillus niger* in flasks of glucose broth with added acetic
acid (top row), with acetic acid and 5 per cent NaCl (middle row), and with acetic
acid and 20 per cent sucrose (bottom row).
  Top row: percentage acetic acid, left to right, none, 0.07, 0.10, 0.16, 0.21, 0.27
  Middle row: percentage acetic acid, left to right none, 0.10, 0.17, 0.23, 0.27
  Bottom row: percentage acetic acid, left to right none, 0.11, 0.16, 0.22, 0.27

per cent and yielded 148 mgm. of dried mold. At a pH 4.5 the
glucose broth brine had an acidity of 0.12 per cent and yielded
127 mgm. of dried mold. This small difference in mold weight
might be attributed to the higher content of acid available for the
mold in the plain broth medium, as the hydrogen-ion concentra-
tion in this case was not sufficient to cause any inhibition of
growth during the first part of the incubation period.

SUMMARY

1. Acetic acid inhibited bacterial growth in almost direct proportion to the amount present. The order of decreasing resistance to acetic acid, either alone, in 5 per cent brine, or in 20 per cent sugar syrup is *Bacillus cereus*, *Salmonella aertrycke*, *Staphylococcus aureus*, and *Phytomonas phaseoli*. Naturally, *Saccharomyces cereviseae* and *Aspergillus niger* were inhibited at a higher acetic acid concentration than the bacteria, with the yeast being the more resistant.

2. At equivalent pH values greater toxicity was usually observed in those tubes containing the greater amount of acid. Apart from the indirect effect in altering the hydrogen-ion concentration, the salt and the sugar aided the acetic acid but little in its toxic effect on bacteria and yeast. Similarly, the added salt and sugar exerted little, if any, effect on the minimum percentage acidity required for total destruction of these organisms.

3. Additional evidence is presented to show that the toxic action of acetic acid on microorganisms is not confined to the hydrogen-ion concentration alone (Levine and Fellers, 1940).

4. When *Aspergillus niger* was inoculated into a series of flasks of glucose broth containing different acetic acid concentrations, growth was inhibited at pH 4.1 and at a total acidity of 0.27 per cent. The addition of either 5 per cent salt or 20 per cent sucrose did not significantly change these limits for growth. The presence of acetic acid in non-toxic amounts actually promoted the development of *Aspergillus niger* by acting as a source of energy.

5. At acetic acid concentrations of 0.10 to 0.17 per cent no inhibition in mold growth resulted from the addition of 5 per cent NaCl. On the other hand the addition of 20 per cent sucrose markedly stimulated growth. Maximum mold growth was obtained in a 20 per cent nutrient sucrose solution at pH 4.4 containing 0.16 per cent acetic acid.

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THE GENUS SPIRILLUM EHBG. WITH SPECIAL REFERENCE TO CELL INCLUSIONS AND THE CHROMIDIAL THEORY

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In previous papers dealing with the structure and life history of *Bacillus mycoides*, *Azotobacter* and *Rhizobium*, the writer (1932, '34, '37, '38) has attempted to show that bacterial cell inclusions, especially fat bodies and volutin balls, have been a principal cause of error in interpretations of cell structure and methods of reproduction.

The present investigation on the genus *Spirillum* is concerned with the cell structure and, especially, with the part cell inclusions may have played in the origin and development of the chromidial theory of the nucleus. The literature contains many papers dealing with the structure of spiral bacteria which, because of their large size, have been favorite objects for cytological study. Since the literature concerning the finer structure of bacteria has been reviewed again and again, and since the several theories of cell and nuclear structure are well known, a further general review is not necessary.

Reviews by Arthur Meyer (1912), Dobell (1911), Guilliermond (1908), and Enderlein (1925) combine to include most of the literature from the earliest period of study through 1925. The more recent work is included in a bibliography by Lindegren (1935).

The theory that the bacterial nucleus consists of chromatin granules, chromidia, diffused throughout the cell plasm has had many advocates and is perhaps the most generally accepted theory at the present time. It is true, however, that this theory has received many adverse criticisms based on cytological evidence and theoretical considerations.

The writer has long believed that much of the support for the theory of a chromidial system is based on the study of species which are known to contain certain cell inclusions which might easily be confused with chromatin bodies.

Since the largest of all true bacteria occur in some species of *Spirillum*, and, since much of the support for the theory of diffused chromatin bodies has been obtained from a study of these forms, this group was chosen for a complete study of the problem.

MATERIAL AND METHODS

An attempt was made to obtain pure cultures of some of the large species from other laboratories and from the American Type Culture Collection, but no such cultures became available. *Spirillum rubrum* Esmarch and *Spirillum virginianum* Dimitroff were procured from the American Type Culture Collection. The cells in these species are rather small and not especially favorable for cytological study.

It is of interest to note that Giesberger (1936), commenting on the matter of cultivation and the lack of pure cultures wrote as follows: "Es will mir vorkommen, dass die Ursache davon zu suchen sei in dem Umstand, dass die Reinzüchtung von Spirillen noch immer eine ungenügend gelöste Aufgabe ist. Kennzeichnend für die unbefriedigende Lage ist wohl in genügenden Masse die Tatsache, dass eine vor wenigen Jahren angestellte Rundfrage an die bekanntesten bakteriologischen Laboratorien der Welt, Reinkulturen von *Spirillum*-Arten zur Verfügung zu stellen, einen fast negativen Erfolg hatte."

Since pure cultures could not be obtained, it became necessary to make original isolations or to proceed with the study from raw cultures in plant infusions. Isolation of pure cultures of spiral bacteria has generally been regarded as a rather difficult matter, although various workers, notably Kutscher (1895) and Giesberger (1936), were successful with relatively simple methods.

Raw cultures containing an abundance of spiral forms were readily obtained in plant infusions prepared from creek and pond waters. Hay or other plant material was added to some of the jars, while others contained only fresh water algae which soon

began to decay when brought into the laboratory and placed in darkness at room temperature. The incubation period required for development of raw cultures rich in spiral forms was found to vary somewhat in the several cultures from different waters. In the early stages of decomposition, spiral bacteria were rarely seen, but as the cultures aged, they became more and more abundant until, at times, the surface pellicle was literally a swarming mass of spiral cells. This condition was generally reached in a period of about one to three weeks. Microscopic examination showed that several distinct types of spiral bacteria were present.

Isolation of pure cultures of some species was found to offer no difficulties. This was the case with *Spirillum serpens* (Müller) Winter, *Spirillum undula* (Müller) Ehrenberg, *Spirillum tenui* (Müller) Ehbg. and a small form identified tentatively as *Spirillum itersonii* Giesberger. All of these species grow well on plain beef-extract peptone agar. In plates streaked with a loopful of inoculum from raw cultures, the colonies can be recognized after 24 hours incubation at room temperature. In some cases, isolation on agar slants was carried out from the 24 hour plates, but more generally after incubation for 36 to 48 hours.

While the isolation of the species listed above was accomplished by the simple routine method just described, it was found impossible to isolate pure cultures of *Spirillum volutans* Ehbg. by any of several methods employed. Even in raw cultures this species occurred but rarely and appears to be a relatively rare organism or one in which conditions for development are more exacting and not always suitable in infusions. It appeared in abundance in but two of the many infusions prepared. One of these was of decaying algae from a pool while the other was a hay infusion prepared from creek water. After three weeks the giant spiral cells were present in great abundance in the surface film of these two cultures which contained also *S. undula* and *S. serpens*. Photographs shown in figures 1, 3, 4 and 5 were prepared from the raw cultures. The organisms continued in vigorous growth for about 10 days when they began to diminish in numbers and eventually disappeared.

Isolation of pure cultures was attempted by means of streaked plates and poured dilution plates. The plating media included beef-extract peptone agar, infusion agar prepared according to Zettnow (1897), Vogt's (1899) pea infusion agar, and tryptose agar. Semi-solid agar was employed in the poured dilution plates. When a loopful of the material containing a great many cells was streaked out on agar and examined microscopically for evidence of growth and multiplication, the large spiral cells could be easily seen lying on the agar surface, but there was never any indication of growth or cell division. Colonies of *S. volutans* Ehbg. were not found in any of the poured dilution plates although other species of *Spirillum* were isolated by this method.

CYTOLOGICAL OBSERVATIONS

Since *S. volutans* Ehbg. is the largest of all true spirilla and a favorable subject for cytological study, my observations on this species will be recorded first. The raw cultures described above furnished an abundance of material and served the purpose almost as well as if pure.

In living unstained preparations, viewed either by bright or dark field illumination, the cell body presents a most striking appearance. The most notable features of the living cells are the enormous size, rapid, flashing rate of motility, and the more or less numerous granules contained in the cell plasm. The granules appear as highly refractive or as dark bodies depending on depth of focus. The cytoplasm is hyaline without any indication of pigment. The granules, as well as the bipolar flagella, are readily seen by dark-field illumination or in films prepared with nigrosin or by Benian's congo red method.

The internal structure is brought out clearly by vital staining methods. When mounted in dilute solutions of methylene blue, the organisms retain motility for some time after the stain has begun to act. As staining proceeds, some granules stain deeply while others are non-stainable. The cytoplasm stains much less intensely than the granules. The stainable granules are volutin, because they become red in staining, are not readily de-stained with dilute sulphuric acid, and dissolve quickly in

hot water. It was noted that volutin is more abundant in some cells than in others. In very young cells the content is less granular and stains more uniformly.

Non-stainable bodies were present in great abundance (figs. 1, 3, 4 and 5). These resisted the action not only of methylene blue but of other anilin dyes including fuchsin, safranin, and crystal violet. However, when treated by fat-staining methods the reaction was positive in all of the methods employed. The bodies were stained with Sudan IV, indophenol blue (Dietrich and Liebermeister method), and by the several methods of Eisenberg (1909). In addition to these well known methods for staining fat bodies in bacteria, the new method of Hartmann (1940) was tested and found highly satisfactory. The new compound, Sudan Black B, stains fat quickly and intensely. The method has certain advantages over the older methods since most of the trouble from precipitates is eliminated, and the slow evaporation rate of the solvent removes the necessity for sealing.

As may be seen from the accompanying photographs of fixed stained films, fat bodies are not always arranged in the same manner in different cells or even in the same cell. Two of the individuals in figure 1 show a single axial row while all the others contain additional bodies which constitute in part a second row. This mode of dispersal is seen best in figures 3 and 5. In figure 5 the cell to the right shows an arrangement which is frequently seen. There are two distinct rows, each lying contiguous to the outer membrane, and so arranged that the individuals in one row alternate with others in the opposite row, thus forming a spiral. The peripheral cytoplasm appears as regular blocks between the fat bodies while the remaining cytoplasm is compressed into thin plates which take the form of a spiral thread. The arrangement is not always perfect, nor does it generally occur throughout the full length of any one cell. Further reference will be made to this arrangement of fat bodies and cytoplasm in a later paragraph concerning the so-called spiral nuclear filament described in other species by some writers.

For the preparation of fixed stained films, suspensions in water were exposed for about two minutes to fumes of osmic acid which

kills the cells quickly. Films were then prepared and air-dried in the usual manner without heating. The air-dried films were then covered with 95 per cent ethyl alcohol which was allowed to act about 5 minutes, after which the films were redried. This method caused very little or no distortion.

Various staining methods were employed for differentiation. Stained by Gram's method, the cells gave up the crystal violet quickly and except for the non-stainable fat bodies took the red color with safranin as the counter stain. Although generally reported as gram-positive, the reaction was found to be negative. Control films of known gram-positive species when mixed in the same film with the spiral cells were strongly positive. There is, therefore, no hesitation in pronouncing this a gram-negative species. The same was found true for all other species of *Spirillum*.

Fixed films stained with Loeffler's methylene blue gave the same differentiation obtained with this dye by vital methods, namely, red-violet volutin balls, colorless fat bodies, and blue cytoplasm. Some films were overstained with methylene blue and then treated with Lugol's iodine solution followed by Vesuvin (figs. 4 and 5). Volutin and cytoplasm were black, while fat bodies remained colorless. The photograph shown in figure 3 was made from a film stained with methylene blue followed by safranin. The volutin balls were blue-violet, cytoplasm red, and fat bodies unstained. The cells in figure 1 were stained by the iron alum haematoxylin method. Volutin and cytoplasm were black, while fat bodies were unstained.

As implied in the descriptions above, none of the formed bodies in the cell could be regarded as chromidia nor as true vesicular nuclei. It appears that, if these large cells contain differentiated chromatin, the substance must be so finely divided and dispersed in the plasm that individual particles are invisible.

Other species of *Spirillum* including *S. undula* (Müller) Ehrenberg, *S. serpens* (Müller) Winters, *S. tenue* (Müller) Ehrenberg, *S. itersonii* Giesberger and *S. virginianum* Dimitroff were examined by the methods described above except that pure cultures were employed. Preservation of these cultures has been insured

by depositing them in the American Type Culture Collection. It may be noted here that pure cultures of the several species have been found quite useful in teaching bacteriology. They are especially suitable for flagella staining, the study of volutin and fat bodies, the rigid nature of the cell wall, and motility.

S. undula is similar to *S. volutans* but on a smaller scale. The typical spiral form is seen best when cultivation is carried out in liquid media. However, for the study of cell structure, agar cultures are preferable because there is less trouble from confusing artifacts. Cells from young cultures contain few or no granular bodies. The content stains uniformly with any of the usual anilin dyes. As the culture ages, characteristic unequal staining occurs, and the appearance which has been so frequently described as banded, barred, vacuolated, beaded, or alveolar is clearly shown. The cells in figure 2 were taken from a 72-hour agar slant culture, killed with fumes of osmic acid, fixed with alcohol, and stained with methylene blue, Lugol's iodine solution, and Vesuvin.

The volutin balls and cytoplasm stained by this method are deep black, while fat bodies remain colorless. It may be seen that some fat bodies equal the diameter of the cell body while others are relatively small. There is also a tendency toward arrangement in a spiral form and for the cytoplasm to appear as zig-zag lines and irregular masses. Since volutin balls are embedded in the cytoplasm, it sometimes happens that the latter is almost completely obscured by the more deeply stained volutin. That the clear gaps are actually due to fat bodies is easily proved by fat staining methods. Volutin was identified by the usual well known methods. The plasm between fat bodies appears to be a substance which is uniform in structure and in staining reaction. It must consist, either of cytoplasm alone, chromatin alone or a finely dispersed mixture of both.

S. serpens (Müller) Winters was found to be the most common species developing in raw cultures as well as the most easily isolated and identified. It grows vigorously on plain broth and agar. Two types of colonies occur on plating from old broth cultures. The typical colony form is striated and could be classed

as the rough type.   Smooth colonies show no markings; they are smaller and more typically dome-shaped than rough colonies.

Cytologically the pattern simulates that of *S. volutans* and *S. undula*.   Fat bodies are not abundant until the cultures have grown 24 hours or longer.   Volutin is present in most cells within 18–24 hours when cultivated on plain beef-extract peptone agar plus 0.05 per cent potassium acid phosphate.   It is true, however, that the number of volutin grains is quite variable in different cells.   In the absence of fat and volutin, the plasm stains uniformly, although less densely than that of *S. undula* and *S. volutans* (fig. 6).

S. tenue, *S. itersonii* and *S. virginianum* are less satisfactory for cytological study because the cells are small.   Each deposits both fat and volutin.   The structure of *S. volutans* Ehbg. is typical for all the species studied, and because of the large size is more easily seen.

DISCUSSION OF CYTOLOGICAL OBSERVATIONS

The only previous cytological study of the true *S. volutans* Ehbg. was made by Bütschli (1902).   He described the cell plasm as a honey-comb or alveolar structure differentiated into a peripheral layer and a more dense "central body."   Because the central portion contained granular bodies which stained red with haematoxylin, he regarded this portion as homologous with a true nucleus.   In view of the structure reported above and illustrated in the accompanying photographs, it seems obvious that Bütschli's red-staining granules were volutin; the alveolar structure was due to fat bodies; and that there is no differentiation into "central body" and peripheral cytoplasm.

Zettnow (1891, 1897, 1899) studied several species of bacteria including *S. undula* and *S. giganteum*.   He confirmed the structure described by Bütschli and introduced the terms *ectoplasm* and *endoplasm*.   He believed that bacteria consist principally, or in some cases entirely, of nuclear substance.   After a long lapse of time, Zettnow (1918), returned to the subject to present new evidence, and to revise his former conclusions.   All of his papers are well illustrated with excellent reproductions of photo-

graphs. In his final paper he concludes that the structures which he formerly designated as chromatin bodies were nothing but grains of volutin, and that chromatin granules, if present, must be so finely dispersed as to be invisible. Rejecting the true nuclei described by Meyer (1912) and others, he regards the theory of finely dispersed chromatin and the theory of a true nucleus too small to be seen as the only possibilities supported by cytological evidence.

Several workers reported on the structure of *S. giganteum* (Kutscher) Migula, and supported the chromidial theory in some manner. Swellengrebel (1907) described spiral or zig-zag "chromatin filaments" and grains of volutin distributed through alveolar, honey-combed cytoplasm. Hoelling (1911) denied the chromatin filaments reported by Swellengrebel, recognized volutin bodies as such, and described the cytoplasm as alveolar. Guilliermond (1908) described the cytoplasm as net-like, recognized volutin as such, and believed that chromidia were also present.

Nakanishi (1901), who reported true vesicular nuclei in many species of bacteria, studied *S. giganteum* and *S. serpens*. He recognized non-stainable granules and regarded them as the cause of the honey-comb structure; noted the presence of stainable granules in both species; failed to identify either type of granule, and regarded them as vacuoles filled with reserve substance. He could find no nucleus in *S. giganteum* but reported a nuclear thread divided into several pieces in *S. serpens*.

Dobell (1911) described nuclei in some unidentified species of *Spirillum* from intestinal contents of frogs and lizards. He thought that the nucleus could occur as a spherical body, several scattered granules, or as a chromatin thread depending on different stages of development. Two of his figures resemble *S. undula* and *S. itersonii*. His position is weakened by the fact that he made no tests to distinguish the so-called chromatin bodies and filaments from volutin, and it was impossible to distinguish sequence of development in the material he examined.

Dimitroff (1926) reported the structure of *S. virginianum* as follows: "Stained specimens show granular (barred) structure

and chromatin spirals in the cytoplasm." He seems not to have recognized cell inclusions, although both fat and volutin are deposited.

Evidence from these studies on spiral bacteria shows that no worker has supported the theory of a definite vesicular nucleus. Bütschli alone advocated the notion of a chromatin containing "central body" homologous in structure with a true nucleus. Zettnow in his early studies confirmed the work of Bütschli but eventually favored the idea of dispersed invisible granules of chromatin. All workers failed to recognize the part played by fat bodies in the so-called honey-comb structure, and with the exception of Swellengrebel, Hoelling, and Guilliermond, confused volutin with chromatin.

A diffuse or chromidial nucleus has been described for many bacillary species as well as for spiral bacteria. There seems no reason to doubt that most if not all of the so-called chromidia were volutin balls, or compressed cytoplasm conditioned by non-stainable fat bodies as shown above.

More recently investigators have sought to prove the presence of thymonucleic acid in bacterial cells by means of Feulgen's reaction. Some workers have reported stainable discrete particles; others saw only diffuse staining of the protoplasm, while still others reported the reaction as negative. The literature has been reviewed by Stille (1937).

CHROMIDIA AS A MECHANISM OF HEREDITY

The theory that chromatin granules variously dispersed in the plasm of bacterial cells perform the functions of a true nucleus as been accepted by many bacteriologists. It has been criticized by others who have maintained that the cytological evidence is at fault, and that the theory fails to provide a mechanism for an orderly transmission of hereditary units.

The advocates of a diffuse chromatin nucleus have held a chemical rather than a morphological conception of the nucleus. They have been satisfied to prove that the bacterial cell contains "nuclear material" in the form of particles of "chromatin" variously dispersed in the cytoplasm. It is obvious that much

of the cytological evidence for a diffuse nucleus is erroneous, and there seems to have been no very clear conception as to the precise manner in which diffused chromidia could function as a nucleus.

The true nucleus has long been known as a morphological unit which behaves in an orderly and uniform manner. It is an entity and a mechanism which performs definite functions, and its manner of functioning is well known. Although all true nuclei contain chromatin, it is now believed that this substance is inert. According to modern theories of cytology and genetics, the genes, not the chromatin, are the essential hereditary material.

The gene material is embedded in chromatin to form chromosomes, which in turn are housed in the nucleus. It is known that individual genes are connected together within a chromosome in a definite linear order, the gene string, and that any alteration in *position* of a given gene results in mutation. Division of chromosomes, nucleus, and cell occurs in such an orderly synchronized manner that each daughter cell receives its full complement of hereditary material.

Application of the conception of gene strings to bacteria has been made by Lindegren (1935) who appears to have pointed to a logical way out of the difficulty. According to his conclusions, the nucleus of bacteria might well enough consist of a single gene string with or without the usual encrustation of chromatin. A naked gene string, although wholly invisible, could perform all the functions of a true nucleus. If the gene string should become encased in chromatin, it might appear as a definite visible body. The smallness of bacteria and transverse division of the cell are not objections. Lindegren has shown, by diagrams drawn to scale, that a space 0.2μ in diameter is sufficient to accommodate a gene string of maximum theoretical size, and provide ample room for transmission of genes to the daughter cells in an orderly, definite manner.

This theory of the bacterial nucleus places emphasis on morphology and behavior but does not require that the nucleus must exist as a visible structure. It is in harmony with the view

long held by bacteriologists, that the bacterial cell must contain some functional equivalent of a true nucleus, but it is opposed to the theory of a diffuse nucleus since the gene string does not exist in a diffused form.

In the light of what we know concerning the cytology of bacteria and the laws of genetics, there seems to be no very good reasons to believe that the bacterial nucleus consists of diffused particles.

SUMMARY AND CONCLUSIONS

Various species of the genus *Spirillum* were isolated from plant infusions by simple plating methods. It was found impossible to isolate *Spirillum volutans* Ehbg. by any of several methods employed. Review of the literature affords convincing evidence that this species has not yet been cultivated in pure culture.

All the species investigated deposit fat bodies and volutin. The alveolar or honey-comb structure frequently described by previous workers is conditioned by non-stainable fat bodies. Chromidia and spiral nuclear filaments described by other workers are regarded as volutin and stained cytoplasm. Whether the plasm contains minute invisible particles of chromatin was not determined.

The theory that the bacterial nucleus may consist of a naked gene string, or a chromatin-encrusted gene string (chromosome) is preferable to the theory of a diffuse chromatin nucleus.

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PLATE I

Fig. 1. Cells of *S. volutans* Ehbg. from raw culture. The cells were killed by fumes of osmic acid, fixed with alcohol and stained with iron alum haematoxylin. The clear areas are fat bodies; cytoplasm and volutin balls are stained black. ×2250

Fig. 2. Cells from pure culture of *S. undula* (Müller) Ehrenberg grown on beef extract peptone agar 72 hours, killed and fixed as above and stained with Loeffler's methylene blue, Lugol's iodine and vesuvin. Cytoplasm and volutin balls are stained black; fat bodies are not stained. ×2250

Fig. 3. *S. volutans* Ehbg. as in figure 1. Stained with Loeffler's methylene blue and safranin. Numerous unstained fat bodies throughout the cytoplasm and a few volutin balls are shown. There is no indication of a "central body." ×1500

Fig. 4. *S. volutans* Ehbg. Cells treated as in figure 1. Stained as in figure 2. Axial unstained fat bodies, volutin balls and cytoplasm are shown. ×2250

Fig. 5. *S. volutans* Ehbg. As in figure 4. The fat bodies are arranged in form of a spiral. The cytoplasm compressed by fat bodies appears as a nodose spiral thread. ×2250

Fig. 6. *S. serpens* (Müller) Winter. Cells treated as in figure 1, stained as in figure 2. Volutin balls, fat bodies and cytoplasm are shown. ×1500
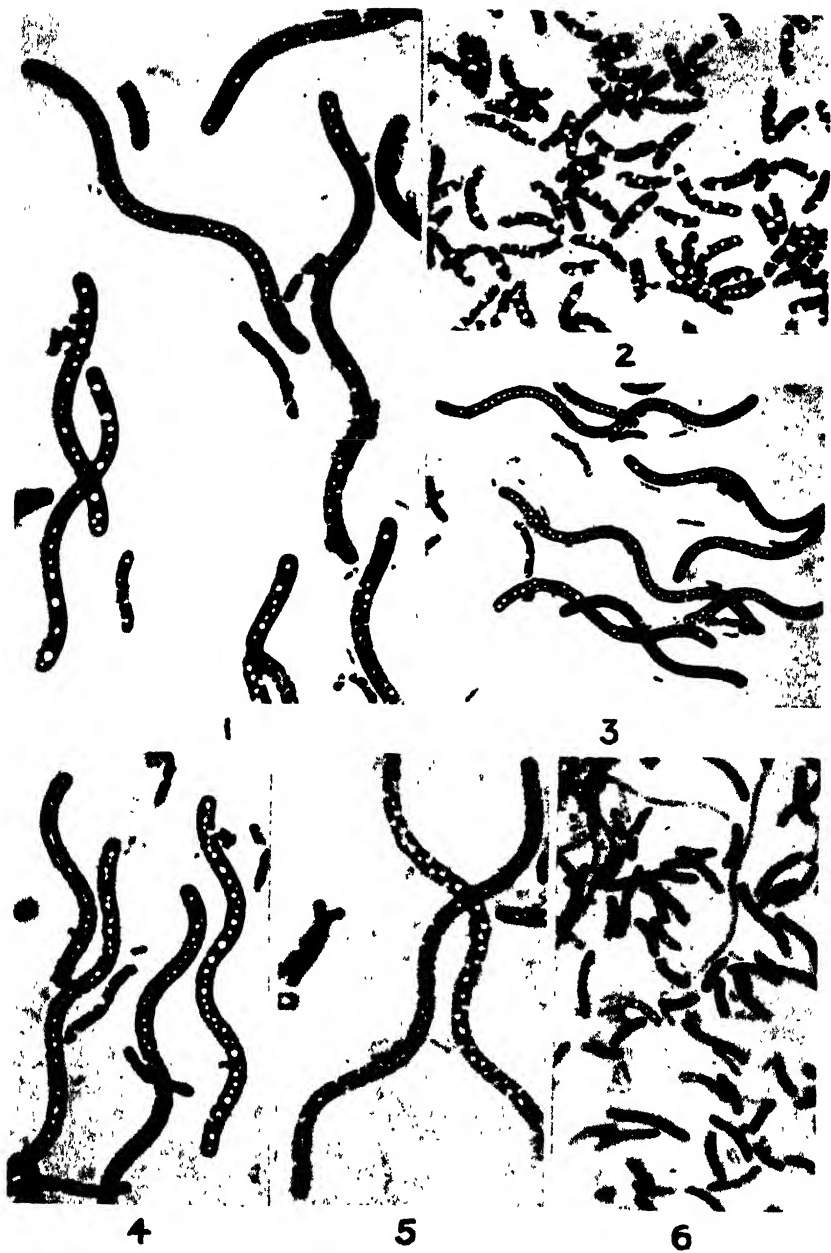
NON-SPORULATING ANAEROBIC BACTERIA OF THE INTESTINAL TRACT[1]

I. Occurrence and Taxonomic Relationships

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This investigation was undertaken mainly to extend the observations of Eggerth and Gagnon (1933) and Weiss and Rettger (1937) on the occurrence of non-spore-forming, obligately anaerobic rod-shaped bacteria in the human intestine. Paths soon led to a study of relationships of the various organisms to each other, ánd of the gram-negative types to certain other closely related bacteria.

A voluminous but scattered literature has accumulated which deals with the isolation and study of non-sporulating anaerobes, not only from the normal intestinal tract, but also from a variety of lesions in the human and animal body (see Weinberg, Nativelle and Prévot, 1937; McCoy and McClung, 1939). Much uncertainty results from a survey of past work, since differences in methods, inconsistent nomenclature, and inherent differences in the organisms themselves have contributed to provide a complex and confusing picture. Do the various pathogens described in the literature represent separate species? Are they distinct from the intestinal forms? And what are the true natural habitats of these organisms? These are questions which arise from examination of previous researches.

The occurrence of these anaerobes in nature may be regarded as being immediately referable to the intestinal and respiratory

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[1] This paper covers in part the Dissertation presented to the Graduate School of Yale University by the senior author as a requirement for the degree of Doctor of Philosophy.

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tracts. They have seldom been observed outside of the animal body, but have been associated frequently with appendicitis (Veillon and Zuber, 1898), colitis (Dack, Heinz and Dragstedt, 1935), genito-urinary disorders (Halle, 1898 and Cotte, 1899), bacteriemia following intense fever (Teissier, Reilly, Rivalier and Layani, 1929), rhinitis (Tunnicliff, 1913), influenza (Olitsky and Gates, 1921 a and b, 1922 a and b, 1923) and other acute respiratory infections (Guillemot, 1899; Olitsky and McCartney, 1923; and Noble and Brainard, 1932), any of which may have their microbial origin in the digestive or respiratory tracts.

Although it seems evident that both pathogenic and saprophytic species of non-sporulating anaerobic bacteria may exist in the intestine, some of the early work on pathogenicity was probably made difficult by the presence of non-pathogenic species which normally occur there. This assumption appears plausible, in view of the findings of Eggerth and Gagnon (1933), who showed that 91 per cent of 60 human stools contained non-sporulating anaerobes as the predominating cultivable bacteria. These results were confirmed by Weiss and Rettger (1937) and Misra (1938).

Little can be gained from a compilation of the characteristics of individual species, for reports have not agreed in detail, and no really satisfactory basis for classification along natural lines has as yet been developed. However, the several attempts to arrange these organisms systematically possess certain merits, although none have proved worthy of general adoption.

Castellani and Chalmers (1919) apparently first tried to group the non-sporulating anaerobic rods in a new genus which was termed *Bacteroides*. Bergey (1923, 1925, 1930, and 1934) and Hauduroy, Ehringer, Urbain, Guillot and Magrou (1937) followed this scheme to a large extent, with modifications intended to accommodate additional species; yet, several similar organisms were distributed by them in other genera, including *Hemophilus*, *Dialister* and *Corynebacterium*.

Rahn (1929) and others have objected to this presentation of the genus *Bacteroides*, because of its extreme heterogeneity and the questionable choice of diagnostic characters.

Weinberg, Nativelle and Prévot (1937) and Prévot (1938) attempted more extensive descriptions of species and their elaborate organization. While they have clarified some of the more general aspects of the problem, confusing and sometimes arbitrary methods of grouping have brought increased complexity of detail to an already badly confused problem.

Eggerth and Gagnon (1933) and Eggerth (1935) recognized the gram-positive intestinal forms as distinct from the gram-negative, but retained the generic term *Bacteroides* as a temporary designation for both. They also recognized numerous species, including 22 new ones, chiefly on the basis of differences in carbohydrate fermentation.

Weiss and Rettger (1934, 1937, 1938) regarded only the gram-negative species as belonging to the genus *Bacteroides*, and emphasized the relationship of the gram-positive forms, especially certain "bifidus" types, to the genus *Lactobacillus*. They were unable to confirm the extensive species differentiation of Eggerth and Gagnon (1933), and placed all gram-negative intestinal forms in four groups, on the basis of agglutination and morphology.

In the fifth edition of Bergey's Manual of Determinative Bacteriology (Bergey, Breed, Murray, and Hitchens, 1939) Roy and Kelly have recognized many of the criticisms of previous classifications. All gram-positive strains were excluded from *Bacteroides*, and several of them were regarded as synonymous with *Lactobacillus bifidus*. Descriptions of 23 gram-negative species are given. In spite of these rearrangements, much doubt appears to remain concerning the systematic relationships of the non-sporulating anaerobes, for Roy and Kelly state that "... it is difficult to know how many should be considered as distinct species, and the present arrangement must be considered tentative."

<div align="center">MATERIALS AND METHODS</div>

Egg-meat medium[3] was employed to maintain stock cultures. For isolation of bacteria from feces and intestinal contents the

[3] Modified formula of Rettger (1906–1907).

following media were used: (1) the beef-infusion medium of Eggerth and Gagnon (1933) prepared with Fairchild peptone and adjusted to pH 7.2, (2) the glucose-cysteine medium of Bedell and Lewis (1938), and (3) the special infusion agar described below. In some instances enrichment with serum, defibrinated blood, glucose, liver infusion and yeast extract was resorted to.

Special infusion agar

Clarified infusion from 2 lb. lean beef....	500	ml.
Mineral salt solution (Allison and Hoover, 1934).....	500	ml.
Fairchild peptone.............................	10	gm.
Savita (Battle Creek Food Co.).........	5	gm.
Glucose.........	0.5	gm.
Potato extract*..........	20	ml.
Serum (human or bovine).......................	20	ml.
Agar.................	20	gm.

Adjust to pH 6.8.

Mix all ingredients; add 30 to 35 ml. of normal NaOH solution; heat in flowing steam for 15 minutes; mix thoroughly; heat in autoclave at 120°C. for 15 minutes; decant clear supernatant portion and discard precipitate; adjust to pH 6.8; sterilize in autoclave at 120°C. for 15 to 20 minutes.

*Potato extract: Mix 100 grams of ground potato with 100 ml. of distilled water and place in the refrigerator over night. Strain through cheese cloth; allow starch to settle, and filter through paper. Add enough NaOH to equal N/5 solution. Heat in autoclave at 120°C. for 20 minutes.

The glucose-cysteine medium and the tomato medium of Weiss and Rettger (1934) were adapted for the determination of morphological, cultural and physiological characteristics.

Broth and agar surface cultures were freed from atmospheric oxygen by the method of Weiss and Spaulding (1937). Stab and shake cultures in deep semisolid agar were employed as such, without additional oxygen exclusion technique.

The various characteristics of the organisms were determined as follows:

Morphology by examination of smears prepared from the surface growths of 48-hour glucose-cysteine agar cultures and stained by the Hucker (1922) gram-staining method.

Motility by the usual hanging drop method and by the Sturges (1928) sealed capillary tube technique. Wet mounts were

prepared also by diluting growth material from 48-hour glucose-cysteine semisolid agar cultures.

Sporulation by the usual heat test, using infusion broth inoculated from 7 day-old egg meat cultures.

Encapsulation by observation of smears stained according to the method of Anthony (1931).

Colony, slant agar and broth culture characteristics by study of 48-hour growths (37°C.).

Gelatin liquefaction by examination over periods of 30 days (37°C.) of gelatin cultures made in the adapted glucose-cysteine medium containing 12 per cent gelatin.

Action on milk in tubes of whole milk containing iron filings and incubated 18 days at 37°C.

Growth temperature ranges by incubation of duplicate serum-tomato-semisolid agar stab cultures at 5-degree intervals covering a range of 5–55°C., and an observation period of 28 days.

Thermal resistance in melted glucose-cysteine semisolid agar tubes inoculated with 0.1 ml. of a 48-hour glucose-cysteine broth culture, and exposed to 60°C. for 5-minute intervals over a period of 30 minutes. Upon removal, tubes were immediately water-cooled until solidified, then incubated at 37°C. for two weeks.

Catalase production by flooding actively growing glucose-cysteine agar plate cultures with 3 per cent solution of hydrogen peroxide.

Indol formation in medium composed of 2 per cent Bacto-tryptone, 1 per cent Bacto beef extract and 0.05 per cent cysteine hydrochloride. After 5 and 12 days of incubation under anaerobic conditions the cultures were tested by layering 0.5 ml. of Kovac's reagent (Ruchhoft, Kallas, Chinn and Coulter, 1931) over 5 ml. of the cultures. The presence of indol was indicated by appearance of a red ring at the surface.

Hydrogen sulphide production in glucose-cysteine semisolid agar stab cultures containing 0.05 per cent basic lead acetate, the observations extending over periods of 33 days.

Nitrate reduction in glucose-cysteine broth containing 0.5 per cent potassium nitrate, by the sulphanilic acid dimethyl alpha naphthylamine method, as recommended by the S. A. B.

Committee on Bacteriological Technique (1937).   Observations were made after 4 and 13 days incubation at 37°C.

Acetyl methyl carbinol formation in 4 days old glucose-cysteine broth cultures, according to the method of Levine (1933).

Fermentation of carbohydrates and alcohols in glucose-cysteine semisolid agar medium minus the glucose and disodium phosphate, as the basal medium.   The test substances were sterilized separately in 10 per cent aqueous solutions and used in a final concentration of 0.5 per cent.   Observations were made after 4 and 16 days incubation at 37°C.   Acid production was determined by mixing one loopful of culture with one drop of Brom-thymol-blue solution and five drops of distilled water on a spot plate.   Gas formation was shown by the appearance of bubbles or torn areas in the agar.   Anaerobic broth cultures in Durham fermentation tubes showed comparable acid production, but gas formation by either method was variable.

In the above-mentioned tests one 4 mm. loopful of 4 to 6 day old egg-meat cultures served as transfer inoculum, with few exceptions.   The cultures were, as a rule, incubated at 37°C. for from 4 to 6 days under anaerobic conditions.   Exceptions are noted as such.

RESULTS

Occurrence of non-sporulating anaerobic bacteria in the intestinal tract

Human feces.   Twenty-one samples of feces from 19 apparently normal adults were examined, and non-sporulating anaerobic organisms isolated from all.   This was accomplished by preparing anaerobic plates and semi-solid agar shake cultures from serial dilutions of each sample, making streak plates from the incubated shake cultures, and incubating all agar plates for from 4 to 6 days.   Delicate small round colonies appearing on the plates were subcultured to special infusion agar slants having long butts by the surface streak and deep stab method.   Cultures showing anaerobic growth only were subjected to the gram stain and heat test for spores.

Of the 550 colonies picked, 162 failed to grow in subculture;

157 were members of other genera (*Streptococcus*, *Clostridium*, *Lactobacillus*, etc.) and 220, or 40 per cent, proved to be non-sporulating anaerobic rod-shaped bacteria. The gram-negative strains obtained in this manner outnumbered the gram-positive forms approximately 4 to 1. We must not assume, however, that this represented their actual frequency ratio in the feces, for differences in nutrient requirements and difficulties in interpreting some of the gram stains altered the figures materially.

Since isolation of these anaerobes was possible only from the higher dilutions, where overgrowth by other bacteria did not occur, it appeared that they must be consistently present in large

TABLE 1

Estimation of demonstrable non-sporulating anaerobic bacteria in human feces and frequency of L. acidophilus

SAMPLE	PER CENT L. ACIDOPHILUS*	NUMBER OF NON-SPORULATING ANAEROBIC BACTERIA PER GRAM OF FECES
C. C.......	70	10,000,000
L. C..	40	10,000,000
S. Y..	85	100,000,000
J. C.......	10	100,000,000
C. P.	0	100,000,000
D. K.	75	100,000,000
B.		1,000,000
S....		100,000,000,000
O..		10,000,000

* Per cent of all visible colonies appearing on the standard tomato agar plates.

numbers. In order to obtain more accurate information on this point nine samples of feces were subjected to quantitative plate counts. Six of these samples were checked by another member of the laboratory staff[4] for the relative abundance of *Lactobacillus acidophilus*, as determined by counting typical rough colonies on tomato agar plates, and estimating them as per cent of the total flora appearing on these plates. As seen in table 1, the number of cultivable non-sporulating anaerobic bacteria varied from one million to one hundred billion per gram of feces, with a

[4] Dr. J. E. Weiss, then Research Fellow in Bacteriology, Yale University.

mean of 100,000,000 organisms per gram; and no correlation existed between the number of these organisms and the incidence of *L. acidophilus*.   We may assume from these observations that, although the non-sporulating anaerobic bacteria are the predominating microorganisms in human feces, the factors which permit their abundant growth in this environment are not identical with those which influence the proliferation of *L. acidophilus*.

Feces and intestinal segments of white rats.   The feces and contents of the stomach, duodenum, jejunum, upper ileum, caecum, upper colon, middle colon and rectum of 25 white rats were collected aseptically in tubes of sterile water.   Ten animals were fed 12 grams of chopped raw lean beef daily for three weeks. Seven were held for two weeks on this regimen; then for a period of three weeks they were given the same food plus 2.5 to 3 grams of lactose daily.   Eight rats were maintained for two and one-half weeks on a diet low in calcium, but otherwise balanced (based on the feeding method of Epright, Valley and Smith, 1937).

Immediately before sampling, the rats were killed by asphyxiation with illuminating gas or ether, and the intestinal segments forthwith removed aseptically.   Cultures were made immediately and isolation carried out as described for human fecal samples.

Only 170 (21 per cent) of the 841 colonies selected from the numerous plates proved to be non-sporulating anaerobic bacteria. The greater difficulty of isolation, as compared with the examination of human feces, was due largely to the frequent occurrence of spreading *Proteus* members and the predominance of lactobacilli in several samples.

Taken as an average, less than one third of the samples yielded non-sporulating anaerobes; however, these organisms were encountered in two or more samples taken from all levels of the intestinal tract.   They were most frequently isolated from the middle colon, and least often from the caecum.

Since the rats used in this experiment formed part of the group also used by Weinstein, Weiss and Gillespie (1938), comparison of the occurrence of non-sporulating anaerobic bac-

teria with their data on the hydrogen ion concentration and
L. acidophilus content of the intestinal segments was attempted,
but no correlation could be established.

When attempts were made to relate the number of segment
samples yielding these anaerobes to diet, pronounced differences
in frequency of isolation were apparent.   Table 2 shows the total
number of samples examined from animals on each diet and the
number from which non-sporulating anaerobic bacteria were
isolated.   The ratios of frequency of isolation were 1:3:8, the
calcium-deficient diet yielding the anaerobes least often, and the
diet of meat plus lactose providing the largest numbers of such
organisms.   The majority of strains isolated from the latter
source were gram-positive and undoubtedly included a few
anaerobic strains of lactobacilli.   In spite of this, it must be

TABLE 2

*Occurrence of non-sporulating anaerobic bacteria in the digestive tract of white rats
and relation of diet thereto*

DIET	TOTAL SAMPLES	NUMBER OF SAMPLES YIELDING NON-SPORU-LATING ANAEROBIC BACTERIA
Meat...	60	12
Meat plus lactose..	67	41
Calcium deficient...	52	4

recognized that the diet of meat plus lactose was especially
favorable for the development of both gram-positive and gram-
negative strains of non-sporulating anaerobic bacteria.

Systematic relationships of isolated organisms.   Seventy-six
representative strains of non-sporulating obligate anaerobes
were selected from nearly 400 stock cultures, which had been
isolated from human feces and intestinal contents of white rats.
Also, 19 strains of related organisms (listed in table 3) were
obtained from other laboratories.   All cultures were replated
to check purity, and further purity tests were made as the taxo-
nomic study progressed.

As a basis for close consideration of the systematic relationships
of the 95 strains used in this study, summarized descriptions of

them are given here under five different types, three constituting one general group (A) and two belonging to a second group (B). Minor differences were observed among individual strains included in any one type, but the presentation of separate data for individual strains would require unduly long and involved statements in this report.

TABLE 3

Non-sporulating anaerobes and related bacteria obtained from other workers

SPECIES	STRAIN	OBTAINED FROM
Lactobacillus acidophilus............	Conant	L. F. Rettger
Lactobacillus acidophilus............	V	Dept. stock
Lactobacillus acidophilus............	XVII	Dept. stock
Lactobacillus bifidus	9	L. F. Rettger
Bacteroides bifidus (Group 1).......	Lucille 1	A. H. Eggerth
Bacteroides bifidus (Group 2).......	Tiffany 3	A. H. Eggerth
Bacteroides biformis	Helen G	A. H. Eggerth
Bacteroides convexus................	Clem 1	A. H. Eggerth
Bacteroides gulosus	Helena	A. H. Eggerth
Bacteroides incommunis	Botko 2	A. H. Eggerth
Bacteroides variabilis.	X35	A. H. Eggerth
Bacteroides varius....	A-1	A. H. Eggerth
Bacteroides vulgatus	Marino	A. H. Eggerth
Bacteroides vulgatus...	40	J. E. Weiss
Bacterium fusiformis...............	B	R. Tunnicliff
Bacterium necrophorum..	ROM	G. M. Dack
Bacterium necrophorum	139	G. M. Dack
Bacterium necrophorum.............	143	G. M. Dack
Bacterium pneumosintes	C-17	P. K. Olitsky

GROUP A

Gram-positive, non-sporulating, non-motile, non-capsulated rods, occurring singly, in pairs and occasionally in short chains; gelatin not liquefied; acetyl-methyl-carbinol, indol and hydrogen sulphide not formed; catalase absent; nitrite usually not produced from nitrate; acid commonly produced from glucose, but not from rhamnose; obligate anaerobes.

Type A-1 (13 strains)

Source: Human feces and rat intestine.

Morphology: Straight rods, 0.5×1–2μ; occur singly, in pairs and short chains; pleomorphic and irregularly stained forms usually absent.

Cultural characters: Glucose-cysteine agar colonies; punctiform, translucent to opaque, greyish-white, slightly raised, glistening, smooth, entire or very finely toothed margin, amorphorus or finely granular.

Glucose-cysteine agar streaks; growth scant, thin, flat, smooth, greyish-white, transparent, filiform or echinulate, moist but less glistening than type A-2.

Glucose-cysteine broth; turbidity slight, evenly distributed and persistent; slight sediment, but no surface growth.

Whole milk plus iron filings; no change except with one strain, which produced an acid curd in four days or less.

Glucose-cysteine gelatin; growth but no liquefaction in 30 days.

Temperature relationships: Resistance to 60°C.; survived more than 30 minutes. Minimum growth temperature, 25–30°C. Optimum

TABLE 4

Action on carbohydrates by 13 strains of non-sporulating anaerobic bacteria belonging to type A-1

NUMBER OF STRAINS	PLAIN MEDIUM	ARAB-INOSE	XYLOSE	GLUCOSE	LAC-TOSE	SU-CROSE	MALT-OSE	TRE-HALOSE	RHAM-NOSE	MAN-NITOL
7	−	−	−	+	−	−	±	−	−	−
2	−	−	−	+	−	+	±	−	−	−
3	−	+	−	+	+	+	+	+	−	−
1	−	+	+	+	+	+	+	0	−	−

+, acid; ±, slightly acid; −, no acid; 0, no growth.

growth temperature, 37°C. Maximum growth temperature, 40–50°C. Usually 45°C. or above.

Fermentative activity: Acid but no gas produced from glucose; no fermentation of rhamnose or mannitol; action on other carbohydrates variable. See Table 4 for characteristic reactions.

Type A-2 (9 strains)

Source: Human feces and rat intestine.

Morphology: Straight rods, 0.7×2–3μ; occur singly, in pairs and short chains; a few curved and clubbed cells observed.

Cultural characters: Glucose-cysteine agar colonies; punctiform, translucent, often with opaque spot in center, greyish-white, raised, glistening, smooth, entire or finely irregular margin often showing faint concentric rings, finely granular.

Glucose-cysteine agar streaks; growth scant or moderate, raised, greyish-white, translucent, beaded, moist, glistening.

Glucose-cysteine broth; abundant turbidity and sediment; no surface growth.

Whole milk plus iron filings; no change except with one strain which produced curd in four days or less.

⌠⁽⁴⁾ Glucose-cysteine gelatin; growth but no liquefaction in 30 days.

Temperature relationships: Resistance to 60°C.; survived more than 30 minutes, except one strain which remained alive only 25 minutes. Minimum growth temperature, 15°C. Optimum growth temperature, 37°C. Maximum growth temperature, 40-45°C.

Fermentative activity: Acid usually formed from glucose, but not from trehalose; gas production common, but irregular; fermentation

TABLE 5

Fermentation of carbohydrates by nine strains of non-sporulating anaerobic bacteria belonging to type A-2

STRAIN NUMBER	PLAIN MEDIUM	ARAB- INOSE	XYLOSE	GLUCOSE	LAC- TOSE	SU- CROSE	MALT- OSE	TRE- HALOSE	RHAM- NOSE	MAN- NITOL
21	−	−	−	+G	−	±	+	−	−	+G
22	−	−	−	+G	−	−	+	−	−	±
32	−	−	−	+G	−	−	+	−	−	−
37	−	+G	+G	+G	−	−	+	−	−	−
51	−	−	−	−	−	−	−	−	−	−
62	−	−	−	+	−	−	−	−	−	−
167	−	±	±	+G	−	−	+	−	±	±G
180	−	−	−	+G	−	−	+	−	−	−
194	−	+G	+G	+	+	+	+G	−	±G	−

+, acid; ±, slightly acid; −, no acid; G, gas.

of other carbohydrates variable (see table 5 for reactions of individual strains).

Type A-3 (4 strains)

Source: Human feces.

Morphology: Branched and straight rods; occur singly, in pairs and in chains; pleomorphism common (x, y, and swollen forms).

Cultural characters: Glucose-cysteine agar colonies; punctiform, translucent, greyish-white, convex, smooth, irregular margin, finely granular except for cluster of coarse granules in the center.

Glucose-cysteine broth; slight turbidity.

Glucose-cysteine gelatin; growth but no liquefaction in 30 days.

Temperature relationships: Resistance to 60°C.; survive not longer

than 15 minutes. Minimum growth temperature, not determined. Optimum growth temperature, about 37°C. Maximum growth temperature, not determined.

Fermentative activity: Acid but not gas produced from arabinose, xylose, glucose, lactose, sucrose, and maltose; no action on trehalose, rhamnose and mannitol.

GROUP B

Gram-negative, non-sporulating, non-motile, non-capsulated rods occurring singly, in pairs, and occasionally in short chains; dense turbidity and some sediment formed in broth; gelatinolytic action variable, but usually absent; nitrate usually not reduced to nitrite; acid and frequently gas formed from glucose, but rarely from mannitol; acetylmethyl-carbinol and catalase absent; obligate anaerobes.

Type B-1 (4 strains)

Source: Rat intestine.

Morphology: Large pleomorphic rods, $1 \times 2\text{--}4\mu$; gram-negative, but stain irregularly and sometimes contain gram-positive granules; filaments 10 to 50 microns in length are common; round or oval structures 4 to 8 microns in diameter may occur in the filaments, or exist free.

Cultural characters: Glucose-cysteine agar colonies: each strain capable of reversible dissociation into two kinds, (a) 1–2 mm. in diameter, thin, flat, greyish-white, dull, translucent, irregularly lobed margin with curled and filamentous internal structure and with many pleomorphic forms, (b) 1–2 mm. in diameter, convex, yellowish-white, opaque, entire, amorphous or slightly filamentous, with few pleomorphic cells.

Glucose-cysteine agar streaks; growth abundant, raised, glistening, moist, opaque, echinulate.

Glucose-cysteine broth; turbidity very dense, sediment viscid or flaky, no surface growth.

Whole milk plus iron filings; no change.

Glucose-cysteine gelatin; abundant growth, but no liquefaction in 30 days.

Temperature relationships: Resistance to 60°C.; survive 10 to 15 minutes. Minimum growth temperature, 10°C. Optimum growth temperature, 37°C. Maximum growth temperature, 40–45°C.

Biochemical tests: Hydrogen sulphide is formed. Indol is not formed. Nitrate is not reduced to nitrite.

Fermentative activity: Acid produced from glucose; gas but no acid formed from basal medium without carbohydrate; acid not formed from lactose, sucrose, maltose, trehalose, mannitol, rhamnose, arabinose or xylose.

Type B-2 (46 strains)

Habitat: Human feces and rat intestine.

Morphology: Short rods, 0.5–0.7 × 0.5–2 microns, with blunt but slightly rounded ends; occur singly, occasionally in pairs and rarely in short chains; small filaments 0.7 × 15 microns common; other pleomorphic or irregularly stained cells present in some cultures, but never abundant.

Cultural characters: Glucose-cysteine agar colonies; punctiform, translucent, greyish-white, slightly raised, glistening, entire or very slightly irregular, amorphorus or finely granular; faint irregular radial markings, scattered coarse granules or delicate concentric rings near margin sometimes observed.

Glucose-cysteine agar streaks; growth moderate, raised, glistening, translucent with some opaque areas, greyish-white, surface slightly uneven, echinulate.

Glucose-cysteine broth; turbidity dense, sediment variable in character and amount, no surface growth.

Whole milk plus iron filings; eleven strains were unable to bring about any visible change; thirty-six strains produced an acid curd in four to eleven days.

Glucose-cysteine gelatin; 11 strains caused liquefaction in 30 days or less; 35 grew, but did not liquefy the gelatin in this period.

Temperature relationships: Resistance to 60°C.; seven strains survived less than 5 minutes, 17 for 5, and 19 for 10 minutes; and 3 strains survived for 15 minutes. Minimum growth temperature, 15°C. Optimum growth temperature, 37°C. Maximum growth temperature, 40°–50°C.

Biochemical tests: Hydrogen sulphide is formed by 9 strains, but not by 37 others. Indol is formed by 26 strains, but not by 20 remaining strains. Nitrate reduced to nitrite by only one strain out of 46.

Fermentative activity: Acid and often gas produced by all 46 strains from glucose, lactose and sucrose; by 45 strains from xylose; by 39 strains from maltose; by 34 strains from arabinose; by 31 strains from rhamnose; by 18 strains from trehalose; and by only one strain from mannitol. Gas, but no acid, sometimes appeared in the basal medium without carbohydrate.

Correlative characters and group relationships

Following the suggestions of previous workers, the gram-positive strains (Group A) were separated from the gram-negative strains (Group B).

All members of Group A were non-sporulating, non-motile, non-capsulated gram-positive rods which required special media (glucose-cysteine or tomato media) for growth. They exhibited variable fermentative capacities and were devoid of proteolytic action on milk, gelatin or egg-meat medium.

Types A-1 and A-2 were straight rods without branching, definitely resistant to a temperature of 60°C., strictly anaerobic and typically unable to coagulate milk. These characteristics, together with those given for the group as a whole, and the fact that they were isolated from the intestinal tract, suggested that they should be regarded for the time being as anaerobic members of the genus *Lactobacillus*.

Type A-1 could be differentiated from type A-2 by the smaller average size of cells, higher minimum growth temperature, less turbidity in broth, and absence of gas formation from carbohydrates. The characteristics of type A-1 were not identical with those of any well recognized species, but the properties of type A-2 were somewhat similar to Eggerth's (1935) descriptions of *Bacteroides lentus* and *Bacteroides biformis*. However, a strain of the last-mentioned species supplied by Eggerth was unable to tolerate exposure to 60°C. for more than five minutes, in contrast to the survival of our strains for 25 to 30 minutes.

Type A-3 differed from the other types in its frequent formation of pleomorphic cells, some of which were branched, and in its failure to survive at 60°C. for more than 15 minutes. This type closely resembled Eggerth's (1935) *Bacteroides bifidus*—Group 2, although our strains were all strictly anaerobic, slightly more resistant to heat, and failed to ferment trehalose.

Lactobacillus bifidus—Type I (Weiss and Rettger, 1938) appeared, in our work, to have more characteristics in common with *Lactobacillus acidophilus* than with the other "bifidus" types. The frequent occurrence of true branching in the "bifidus" types suggested a relationship to the *Actinomycetales*. For this reason

the terminology of Prévot (1938) might well be employed to avoid further confusion. On this basis type A-3 and related forms described by Eggerth and by Orla-Jensen would be named *Bifidibacterium bifidum* (Orla-Jensen) Prévot (1938).

Group B consisted of non-sporulating, non-motile, non-capsulated, gram-negative rods which showed no growth on plain nutrient agar, and rather delicate colonies even on special media, such as glucose-cysteine or glucose-infusion-blood agar. Fermentative and proteolytic activities were variable.

Type B-1 was clearly distinguishable from type B-2 in that the cells and colonies were larger, and the cells more pleomorphic,

TABLE 6

Varieties of type B-2 distinguished from each other by physiological characters

NUMBER OF STRAINS	HYDROGEN SULPHIDE	INDOL	TREHALOSE	RHAMNOSE	ARABINOSE
12	−	+	+	+	+
11	−	+	−	+	+
8	+	−	−	−	−
3	−	−	+	−	+
2	−	−	−	+	+
2	−	−	−	−	−
2	−	−	+	+	+
2	−	−	−	+	−
2	−	+	−	−	+
1	+	−	−	+	+
1	−	+	+	−	+

and in that it grew at a lower temperature and failed to ferment either lactose or sucrose. The strains belonging to this type resembled *Bacteroides funduliformis* as described by Henthorne, Thompson and Beaver (1936), except for their inability to ferment maltose and failure to form indol.

Type B-2 constituted a somewhat heterogeneous group of organisms previously described as several distinct species of the genus *Bacteroides*. Lack of correlation among any of the variable characters studied made it impossible to obtain clear-cut subdivisions of this sub-group, although it was possible to detect the three predominating varieties shown in table 6, by use of

hydrogen sulphide and indol production, in conjunction with fermentations of trehalose, rhamnose and arabinose. Unfortunately, about one third of the strains were separated into 8 additional varieties by this procedure, and when other characters, such as gelatin liquefaction or fermentation of additional carbohydrates, were considered, notable differences within varieties became apparent.

Because the significance of species differentiated on such uncorrelated characters is doubtful, it seemed more desirable to recognize the marked similarities among strains in habitat, morphology, cultural characters and certain physiological characters, such as inhibition by molecular oxygen, special nutrient requirements and fermentation of glucose, lactose and sucrose. Until more satisfactory criteria for classification have been developed, type B-2 may well be designated as a single species, *Bacteroides vulgatus* (Eggerth and Gagnon).

All of the *Bacteroides* strains obtained from Eggerth and from Weiss appeared to be closely related to, or identical with, type B-2, although none of our cultures were exactly like Eggerth's *Bacteroides varius* or *Bacteroides variabilis*.

The strains of *Bacterium necrophorum*, *Bacterium pneumosintes* and a member of the *Fusobacterium* genus obtained from other workers were similar to group B in being gram-negative, nonsporulating, anaerobic, rod-shaped bacteria, but physiologically they appeared quite different. When cultured according to the methods used for our own strains, growth either failed to appear or was so slight as to make the results uncertain.

Further attempts to show definite relationships between the intestinal non-sporulating anaerobes and the oral fusiform bacteria were disappointing. The pointed cells noted by Hine and Berry (1937) in their study of the fusiform genus were not characteristic of any of our cultures. Nor were the biochemical groupings observed by Spaulding and Rettger (1937) evident among our strains, even when their media were employed. Much more research is required to determine the exact relationships between these different groups of gram-negative non-sporulating, anaerobic rod-shaped bacteria.

SUMMARY AND CONCLUSIONS

Non-sporulating anaerobic rod-shaped bacteria occur in ex-
tremely large numbers in human feces, often to the extent of
dominating all other demonstrable flora.   They must be regarded,
therefore, as deserving further intensive study, particularly from
the standpoints of taxonomy and the possible rôle that they may
play in the physiology of the intestinal tract.

They were observed also in the various segments of the intes-
tine of the white rat, their numbers being increased materially
by the addition of lactose to a basal meat diet.

There is apparently no definite correlation between numbers of
non-sporulating anaerobes and lactobacilli of the acidophilus-
bifidus type in the human and rat intestine.

Seventy-six strains selected from nearly 400 cultures of human
and rat origin were divided conveniently into two groups, the
first of which (Group A) is gram-positive and consists of three
different types, while the second (Group B) is gram-negative and
is composed of two types.   Types A-1 and A-2 may be, for the
moment, regarded as anaerobic members of the genus *Lacto-
bacillus*, on the basis of certain similarities in morphology, tem-
perature relationships and fermentative activity.   Type A-3
closely resembles *Bacteroides bifidus*, Group 2 (Eggerth, 1935)
which, because of the common occurrence of branched cells, and
certain other characteristics, may be included in the *Actinomy-
cetales* as *Bifidibacterium bifidum* (Orla-Jensen; Prévot, 1938).

Type B-1 shows much similarity to *Bacteroides funduliformis*.
Type B-2 includes different organisms previously described as
separate and distinct species of the so-called *Bacteroides* genus,
but lack of correlation due to variable characters throws doubt
upon the validity of such differentiation; until more satisfactory
means of classification are available these organisms may con-
veniently be designated as a single species, *Bacteroides vulgatus*
(Eggerth and Gagnon).

Group B in certain respects resembles *Bacterium pneumosintes*,
Bacterium necrophorum and the oral fusiform bacteria (Fuso-
bacterium genus), but differences in morphology, growth require-
ments and biochemical activities appear to separate them from

the non-sporulating anaerobes of intestinal origin studied in the present investigation.

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NON-SPORULATING ANAEROBIC BACTERIA OF THE INTESTINAL TRACT[1]

II. Growth-Facilitating Factors

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The statements of Castellani and Chalmers (1919) and Bergey (1934) that the *Bacteroides* show good growth on ordinary laboratory media have been quite misleading, for difficulty of cultivation has been among the most trying problems encountered in working with the non-sporulating anaerobic bacteria. Harris (1901–1905) and Beaver, Henthorne and Macy (1934) have especially emphasized this point, and several other workers have considered survival of pure cultures for more than a month or two as worthy of special comment.

There has been little agreement among investigators concerning the conditions best adapted for the growth of these organisms, and little information is available as to which materials actually contain nutrients available for their use. On a more or less empirical basis, complex infusions, body fluids and tissues have been used in a great variety of formulae. Further inconsistencies have arisen from the use of numerous kinds of peptones, and of reactions varying from distinctly acid to markedly alkaline. The usefulness of most of these media has been limited by their lack of clarity, heat lability and complexity. Often growth appears to have been delicate, slow to appear, and difficult to observe.

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[1] This paper covers in part, the Dissertation presented to the Graduate School of Yale University by the senior author as a requirement for the degree of Doctor of Philosophy (1939), and the thesis submitted to the Graduate School of the University of Nebraska by the second author as a requirement for the degree of Master of Arts (1938).

This investigation was designed to provide definite information regarding the relationships of temperature, gaseous environment, hydrogen ion concentration, and nutrients to optimum growth of the non-sporulating anaerobes of intestinal origin. Special emphasis was placed upon the development of an improved and simplified culture medium.

PROCEDURE

Selection of cultures. Strains were selected from nearly 400 stock cultures previously isolated from intestinal contents of man and rats, and from those supplied by other workers.[2] Although the number of strains used in any one experiment varied from 5 to 162, an attempt was always made to choose representative organisms. Checks on purity were made periodically throughout the investigation.

Preparation of media. Since the constituents of the media were varied widely for experimental purposes, their nature will be indicated with the results. An aseptic tubing method similar to that described by Riker and Riker (1936) was employed when addition of heat-labile substances required that sterilization precede dispensing into tubes. Following incubation of media thus prepared, usually none and never more than one per cent of the tubes showed contamination. When it was necessary to avoid heating, sterilization was effected by passage through Berkefeld W or N filters.

Inoculation and incubation. Four-day-old egg-meat cultures were employed as the source of inoculum. Anaerobic conditions were obtained either by the method of Weiss and Spaulding (1937) or the use of deep cultures in semisolid (0.3 per cent) agar. Incubation at 37°C. was maintained for at least two weeks, unless otherwise specified.

Estimation of growth. Growth was judged by (1) the relative rapidity and abundance of multiplication, (2) the degree of viability on transfer through four successive subcultures of a given

medium, (3) the smallest amount of inoculum necessary to initiate development in a given time interval, or (4) plate counts.

Temperature. Growth temperature relationships of 162 strains were determined by incubating semisolid tomato agar (Weiss and Rettger, 1934) stab cultures containing 10 per cent sterile bovine serum at five degree intervals covering the range from 5° to 55°C. Observations were made through a period of 28 days.

RESULTS

Growth temperature relationships

In table 1 are listed for each temperature the number of strains growing, average days of incubation preceding visible growth and the average distance below the surface at which growth appeared.

TABLE 1

Growth temperature relationships of 162 strains of non-sporulating anaerobic bacteria

TEMPERATURE OF INCUBATION	NO. OF STRAINS GROWING	AVERAGE INCUBATION BEFORE GROWTH APPEARED	AVERAGE HEIGHT OF MEDIUM ABOVE GROWTH
°C.		days	mm.
5	0		
10	4	14.3	24
15	143	6.0	13
20	148	3.5	12
25	159	3.5	11
30	162	1.2	5
35	162	1.0	6
40	162	1.0	6
45	88	1.3	6
50	6	3.5	12
55	0		

The optimum growth temperature was between 35° and 40°C., as judged by the average daily growth of all strains at both of these temperatures.

The minimum growth temperature for the majority of strains was between 15° and 20°C., although the four gram-negative strains previously described as type B-1 developed slowly at 10°C., while the gram-positive strains belonging to type A-1 were unable to multiply below 25° to 30°C.

The maximum growth temperature was less easily determined, because it was often relatively close to the optimum temperature. Undoubtedly it fell slightly above or below 45°C. for most strains, since all of the 162 grew visibly at 40° in one day (average), 88 developed at 45° in 1.3 days, and only six showed growth at 50°C. after 3.5 days.

It was not clearly evident from these data whether the marked differences in nearness of growth to the surface of the medium represented alterations in oxygen tolerance with change in temperature, or were due to variations in the depths to which oxygen diffused into the medium during different periods of incubation.

Gaseous environment

Although atmospheric oxygen has been known to inhibit growth of these non-sporulating anaerobes completely, little information was available concerning the effect of the carbon dioxide and hydrogen used in the anaerobic jars.

A definite stimulating effect of carbon dioxide on the development of bacteria was recorded by Valley and Rettger (1927) and by others, but the earlier studies did not include non-sporulating anaerobes. Eggerth and Gagnon (1933) noted that some of their *Bacteroides* strains were favored by the addition of carbon dioxide, but presented no experimental evidence to support this observation.

To determine the influence of carbon dioxide on the growth of five strains, triplicate plate cultures, using glucose-cysteine agar (formula in section on nutrient requirements) or glucose-beef infusion agar plus 10 per cent potato extract, were prepared from quantitative serial dilutions of egg-meat cultures. One jar was freed from carbon dioxide by evacuating and flushing with hydrogen three times, then filling the jar with hydrogen. The second jar was evacuated and then filled with a mixture of 10 per cent carbon dioxide and 90 per cent hydrogen, as calculated from manometric readings. The third jar was freed from all gases except carbon dioxide by evacuating and filling with this gas three times; finally carbon dioxide was introduced into the evacuated jar to 10 per cent capacity.

Palladinized asbestos was placed in the first two jars to remove remaining traces of oxygen; pyrogallic acid and sodium hydroxide were used in the third jar for the same purpose.

After four days of incubation at 37°C. the colonies were counted. In table 2 are recorded the estimated numbers of organisms per milliliter of inoculum. When carbon dioxide was excluded, four strains failed to grow, and the fifth showed only very slight colony development. In contrast to this, counts ranging from about sixteen million to nearly three billion organisms were found for all strains when plates were incubated in jars containing a mixture of 10 per cent carbon dioxide and 90 per cent hydrogen. The somewhat lower counts obtained when 10

TABLE 2

Influence of carbon dioxide on growth of five strains of non-sporulating anaerobic bacteria

STRAIN NUMBER	ORGANISMS PER ML. OF INOCULUM AS INDICATED BY PLATE COUNTS		
	CO_2 H_2 0 100%	CO_2 H_2 10% 0*	CO_2 H_2 10% 90%
4	0	188,000,000	2,960,000,000
30	0	412,000,000	529,000,000
31	0	111,000,000	2,300,000,000
186	0	51,200,000	51,200,000
196	40,000	12,800,000	16,300,000

* Carbon dioxide to 10 per cent capacity of jar.

per cent carbon dioxide was used alone probably were due to less complete anaerobiosis secured by the use of pyrogallic acid and sodium hydroxide, or absorption of the gas by the alkali alone.

When plate cultures which had been kept in a carbon-dioxide-free atmosphere were incubated in the presence of 10 per cent carbon dioxide and 90 per cent hydrogen, normal colony development took place, thus showing that the absence of carbon dioxide was inhibitory rather than lethal in its effect.

Four repetitions of this experiment yielded similar results and adequately demonstrated that, although hydrogen had little effect on the development of the non-sporulating anaerobic bacteria, they did require carbon dioxide for initiation of growth.

Hydrogen ion concentration

Opinions of previous workers have varied widely regarding the optimum hydrogen ion concentration for growth of the non-sporulating anaerobic bacteria. Eggerth and Gagnon (1933) and Weiss and Rettger (1937) claimed that moderate alkalinity (pH 7.6 to 7.8) was most favorable for initiation of growth, but the latter workers also noted that slight acidity improved the growth of pure cultures of gram-negative species.

To determine the effect of different hydrogen ion concentrations on viability, 16 strains, including three obtained from the above-mentioned authors, were inoculated into a series of glucose-cysteine-semisolid agar (formula in section on nutrient require-

TABLE 3

Influence of hydrogen ion concentration on growth and survival of 16 strains of non-sporulating anaerobic bacteria

pH	NUMBER OF STRAINS SURVIVING 4 TRANSFERS	AVERAGE DAYS OF INCUBATION PRECEDING GROWTH	PER CENT RECOVERY AFTER 30 DAYS
3.7	0		
5.3	4	4	40
6.0	15	3	67
7.0	16	1	81
7.8	11	3	82
8.4	9	3	100

ments) stab tubes which had been adjusted to pH 3.7, 5.3, 6.0, 7.0, 7.8 and 8.4. As soon as growth appeared in any culture tube a transfer was made to another agar tube having the same hydrogen ion concentration. This procedure was repeated until the strains either failed to grow or had been carried successfully through four serial transfers. After incubating the fourth transfers for one month, each tube was inoculated into a medium adjusted to pH 7.0, to determine the relative degree of survival on prolonged incubation in acid and in alkaline media.

Table 3 shows that, on the basis of the number of strains surviving the fourth transfer and the average period of incubation required for development, the optimum hydrogen ion concentra-

tion was between pH 6.0 and 7.0, with a slight balance of evidence in favor of the latter reaction.

The failure of all strains to multiply at pH 3.7, and the survival of only four strains at pH 5.3 indicated that the maximum acidity which permitted growth of the majority of strains lay near pH 5.0.

No medium sufficiently alkaline to prevent growth of all strains was employed; but the survival of only nine strains at pH 8.4, and the three-day incubation period required for their development, suggested that this reaction approached the maximum basicity permitting growth.

When the percentage of strains remaining viable after 30 days was used as the criterion of optimum hydrogen ion concentration, somewhat different conclusions were reached, for the proportion of viable strains steadily increased from 40 per cent at pH 5.3 to 100 per cent at pH 8.4, thus indicating that alkaline media favored continued viability to a greater degree than did neutral or acid media.

A second experiment, employing hydrogen ion concentrations of pH 6.1, 6.3, 6.8, 7.2 and 7.4, confirmed the above observations regarding the difference between optimum pH values for initiation of growth and for survival. It also indicated that near-optimum growth could be obtained over a range of pH 6.3 to 6.8.

In short, these data showed that neutral or slightly acid (pH 6.3 to 7.0) media were most favorable for initiation of growth, but that moderately alkaline (pH 7.2 to 8.4) media sustained viability over a longer period.

Perhaps the apparent differences between these findings and those of Eggerth and Gagnon (1933) and Weiss and Rettger (1937) may be due to the relatively long incubation periods (4 to 10 days) which preceded their observations.

Nutrient requirements

A complete review of the many experiments required to devise a suitable culture medium is inadvisable here. The information presented is a summary of the nature and scope of the investigation, supported by selected data to show points of major interest.

A survey of the nutrient qualities of a variety of previously used media, as well as individual constituents, was made to determine which substances, either alone or in various combinations, were actually responsible for growth.   The materials used may be grouped as follows:

Mineral salts	Carbohydrates and alcohol	Infusion
Sodium chloride	Glucose	Beef
Di-sodium phosphate	Lactose	Liver
Salt mixture (Allison and Hoover, 1934)	Sucrose	Heart
	Mannitol	Veal

Extracts	Protein-like substances
Beef	Gelatin
Yeast	Sodium caseinate
Savita	Egg albumin
Potato	Serum
Fecal	Peptonized milk
Tomato	Peptone

The substances which individually yielded the most promising results when tested in peptone agar were yeast extract, liver infusion, glucose and peptonized milk.   Others, especially tomato juice, serum and veal infusion, were of some value when added to more complex mixtures, such as North gelatin agar (North, 1909; modified by Spray, 1929) or tomato agar (Kulp 1927; modified by Weiss and Rettger, 1934).   Variation in nutrient requirements of individual strains indicated that more than one factor was responsible for growth, and that some of these substances were present in a number of the materials commonly employed in bacteriological media.

From these observations the possibility of modifying plain nutrient agar to support the development of the non-sporulating anaerobes was suggested.   Table 4 shows the progress of this work, beginning with repeated failure to grow the organisms in ordinary nutrient agar containing 0.3 per cent Bacto beef extract and 0.5 per cent Bacto peptone (Medium A).

A concentrated nutrient agar (Medium B) containing five times the usual amount of beef extract and ten times that of ordinary peptone supported initial development of 10 strains in an average

period of three days, but only four strains survived in serial transfers.

A modified nutrient agar containing one per cent each of beef extract, peptone and tryptone (Medium C) was, in itself, no better than ordinary nutrient agar, for all strains failed to grow. However, the success of Spray (1936) in using tryptone for the cultivation of spore-forming anaerobes made it seem advisable to continue to employ this formula as a basal medium for the investigation of other materials.

TABLE 4

Influence of various nutrient media on the growth of 11 strains of non-sporulating anaerobic bacteria

MEDIA	NUMBER OF STRAINS SHOWING GROWTH	AVERAGE INCUBATION BEFORE GROWTH APPEARED
		days
Nutrient agar (A)	0	
Concentrated nutrient agar (B)	10	3
Nutrient tryptone agar (C)	0	
Filtered glucose + (C)	7	7
Autoclaved glucose + (C)	11	3
Cysteine* + (C)	11	2
Cysteine* and glucose + (C)	11	1

(A) Bacto beef extract, 0.3 per cent; Bacto peptone, 0.5 per cent.

(B) Bacto beef extract, 1.5 per cent; Bacto peptone, 5.0 per cent.

(C) Bacto beef extract, 1.0 per cent; Bacto peptone, 1.0 per cent; Bacto tryptone, 1.0 per cent.

* As hydrochloride.

Glucose, when sterilized by filtration and then added to medium C in one per cent concentration, allowed seven strains to grow sparsely in seven days (average). Heat sterilization of the glucose in the medium brought about some change which further improved its growth-promoting properties to the extent that the entire group of 11 strains developed in three days. Whether this involved decomposition of the glucose to more readily available compounds, or a combination of the glucose with other substances in the medium, was not determined.

The use of cysteine hydrochloride was suggested by the work

of Valley (1929), Spaulding and Rettger (1937) and others who had successfully employed it for growing various anaerobic bacteria. When 0.05 per cent cysteine hydrochloride was added to medium C it encouraged all strains to develop in approximately two days.

Addition of both glucose and cysteine hydrochloride stimulated development still further, all strains showing visible growth in about one day.

Further studies on favorable concentrations of the various constituents employed in medium C plus glucose and cysteine showed that the original amounts were at or near optimum, but that some variation in proportions of the constituents, especially glucose, could be made without markedly affecting growth. Comparison of Bacto peptone, Fairchild peptone, neopeptone, proteose peptone, Parke Davis peptone and tryptone revealed that the tryptone supported more rapid and abundant growth than the other peptones employed.

On the basis of these results a medium designated as the *glucose-cysteine medium* was prepared as follows:

Tryptone (Bacto).................	20	gm.
Beef extract (Bacto)........	10	gm.
Glucose	5–10	gm.
Cysteine hydrochloride	0.5	gm.
Di-sodium phosphate (hydrate)..	4	gm.
Distilled water..	1,000	ml.

Dissolve all ingredients and adjust to pH 7.4. For solid medium add agar. Sterilize in the autoclave at 120°C. for 20 minutes.

Adjustment to pH 7.4 was necessary to give the desired final hydrogen ion concentration, namely pH 6.3 to 7.0, after heating. Addition of di-sodium phosphate provided a buffer against the acidity resulting from the fermentation of glucose.

Successful cultivation of more than one hundred of our own isolations, as well as related organisms of the *Bacteroides, Lactobacillus* and *Bacterium* types obtained from other workers, supplied adequate evidence that the glucose-cysteine medium was suitable for growing pure cultures of the non-sporulating anaerobic bacteria of intestinal origin.

Since all strains used had been maintained on laboratory media for more than a year, a further check on the value of this formula was made by comparing the ease with which these anaerobes could be isolated from feces when plated on beef infusion-glucose-blood agar (Eggerth and Gagnon, 1933) and on the adopted glucose-cysteine agar. Fifty-eight per cent of 100-odd colonies originating from three human fecal samples proved to be non-sporulating anaerobes regardless of which medium was employed, thus indicating that the two formulae were of equal value for isolation purposes. When the glucose-cysteine medium was enriched with liver infusion, yeast extract and tomato juice,[3] the proportion of non-sporulating anaerobes to the total recovered rose to 70 per cent.

In short, the glucose-cysteine formula may be recommended as an improved and simplified medium for the cultivation of the non-sporulating anaerobic bacteria of intestinal origin; it supports growth adequately, is comparatively simple in composition, easily prepared, adaptable to a variety of laboratory manipulations, stable at temperatures of the autoclave, and is practically free from turbidity. Furthermore, it is composed of readily available and fairly inexpensive materials.

SUMMARY

Growth of the non-sporulating anaerobic bacteria of intestinal origin is favored by:

1. An incubation temperature of 35 to 40°C.
2. The presence of 10 per cent carbon dioxide.
3. A hydrogen ion concentration of pH 6.3 to 7.0.
4. The improved and simplified glucose-cysteine agar medium described in this paper.

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ASPERGILLOSIS IN WILD HERRING GULLS

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Pulmonary infections with *Aspergillus fumigatus* are well known in domestic fowls or wild birds confined in zoölogical gardens, but the occurrence of such an infection in wild Herring Gulls (*Larus argentatus* var. *smithsonianus*) seems worthy of mention.

During October, 1939, it was reported that gulls in the region of the East Boston airport were afflicted with a fatal disease. This airport is situated on low ground which projects into Boston Harbor. Its outer portion is overgrown with tall grass and weeds, and in this season has several shallow pools containing decaying vegetation. These pools are filled by rain, but become flats of caked mud in dry weather. Investigation of this area revealed the presence of about 20 dead gulls and a flock of about 200 live birds. When approached the flock rose slowly: several birds had difficulty in getting into the air. An agent of the S. P. C. A. stated that he had removed more than 60 birds which were dead or moribund. Three birds which were too weak to fly were examined. They showed emaciation and extreme weakness, but no specific neurological signs or evidence of trauma.

Autopsies were performed on five birds soon after death. All showed lesions of the lungs. In two birds, no lungs could be identified grossly. Instead, there were spaces lined with granulomatous tissue and tubercle-like nodules. The cavities showed many greenish-grey patches of mold, which gave off clouds of spores when touched. These birds also had tubercle-like patches of mold in the peritoneal cavity and on the liver, and a sporulating

colony was found on one kidney. In three other birds, the lungs showed necrotic areas and a watery greenish exudate. Pure cultures of the mold were grown on Sabouraud's agar incubated at 37°C. and identified as *Aspergillus fumigatus*. A culture was sent to Dr. Charles Thom, of the United States Department of Agriculture, to whom we are indebted for confirmation of the identification. No organism was recovered from the heart's
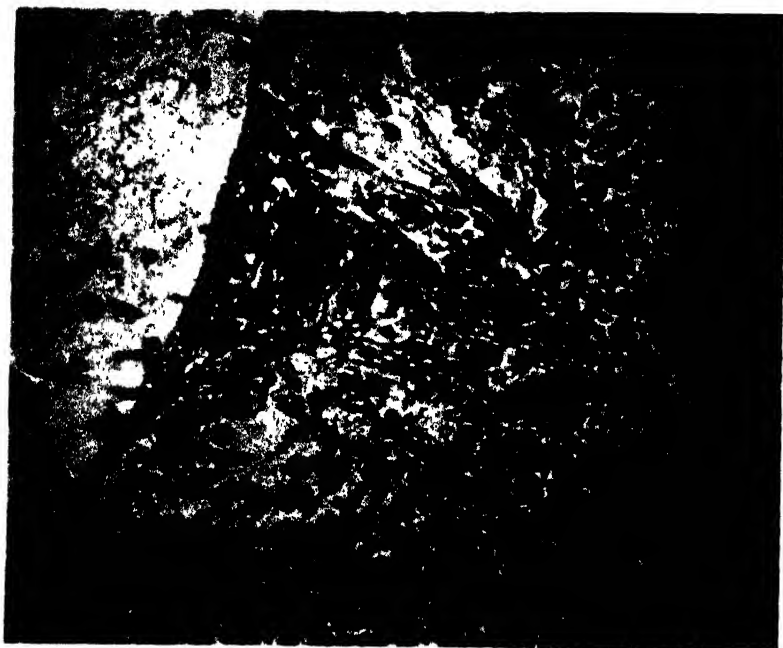
FIG. 1. SECTION OF LUNG OF HERRING GULL (LARUS ARGENTATUS VAR SMITHSONIANUS) STAINED WITH HEMATOXYLIN EOSIN

Mycelia of *Aspergillus fumigatus* are present in the necrotic tissue and are invading an alveolar space. Magnification × 900

blood or brain cultured on blood agar, and, similarly, intracerebral inoculation of Swiss mice and sparrows with heart's blood and brain revealed no pathogenic agent in these tissues. Sections of the lungs showed acute granulomatous lesions with central necrosis containing mycelia (fig. 1). A subacute inflammatory reaction surrounded these areas of necrosis with infiltration of many mononuclear cells and polymorphonuclear leucocytes. Sections

of the bronchi also showed similar inflammatory response. Zenker's fixed sections stained with Ziehl-Neelson's carbol fuchsin failed to reveal the presence of tubercle bacilli.   Thus we conclude that this outbreak constitutes an example of aspergillosis in wild fowl.

Although *Aspergillus fumigatus* is ubiquitous in nature, a history of unusual exposure to decomposing vegetation is usually obtained in cases of aspergillosis (Henrici, 1930; Thom and Church, 1926; Hutrya and Marek, 1913).   Human infection occurs chiefly in the "gaveurs des pigeons" of Paris, or in those exposed to moldy flour.   The disease is commonly found in fowls which have been given moldy straw for bedding, or which are confined to pens with decaying vegetation.   Nearly all the infected gulls were in the second or third-year plumage.   Gulls of this age spend the summer in Boston Harbor, feeding at garbage dumps and serving as general scavengers along the shore. Workmen at the airport stated that flocks of gulls spent the night in the grass; this statement was confirmed by the abundant feathers and droppings among the grasses and around the pools. Thus it is possible that the birds had been exposed to decaying vegetation for several months, both at their feeding place and at night.

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PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

EASTERN PENNSYLVANIA CHAPTER

AN AGENT ISOLATED FROM A SOIL BACILLUS WHICH INHIBITS CAPSULE FORMATION OF FRIEDLÄNDER'S BACTERIUM AND IS HIGHLY BACTERICIDAL FOR GRAM-POSITIVE MICRO-ORGANISMS. *J. C. Hoogerheide*, Biochemical Research Foundation of the Franklin Institute, 133 South 36th St., Philadelphia, Pa.

Progress has been reported in the purification and identification of an agent, isolated from a soil bacillus, inhibiting encapsulation of Friedländer's bacterium. The active agent is soluble in alcohol, acetone, dioxane, pyridin, ethyl-acetate, chloroform, but insoluble in ether, petroleum-ether, benzene and carbon tetrachloride.

Absorption spectra and several positive protein reactions indicate the presence of tryptophane in the molecule in considerable amount (about 25%). A concentration as low as 0.005 mgm. per ml. of culture medium is sufficient to prevent encapsulation.

When tested on inhibition of capsule formation of pneumococci it was found that the same agent, in minute concentrations, has an additional important property, namely that it is highly bactericidal for pneumococci as well as for other gram-positive micro-organisms (Streptococci, staphylococci, *Bacillus anthracis, Gaffkya tetragena, Clostridium sporogenes, Clostridium histolyticum, Clostridium welchii* and *Mycobacterium phlei*).

The active principle injected into mice gave protection against thousand-times lethal doses of pneumococci or anthrax bacilli. Promising results were obtained by treatment of 10 cases of badly infected wounds with solutions of the bactericidal agent.

Similar bactericidal substances could be isolated from other very common aerobic spore-formers (*e.g., Bacillus subtilis, Bacillus megatherium*). Strains excreting such bactericidal substances can be isolated from soil without any pretreatment or adaptation.

HEMOPHILUS PERTUSSIS ANTIGENICITY. *C. Roos and H. P. Bellew*, Mulford Biological Laboratories, Sharp and Dohme, Glenolden, Pa.

The antigenicity of Phase I *Hemophilus pertussis* suspension (Pertussis Bacterin) and extract (Pertussis Topagen) was determined by mouse protection tests.

Mice were given three intraperitoneal injections of 1,000 million organisms (suspension), or their equivalent of extract as determined by the complement fixation test. They were tested for immunity three weeks after the last dose, by the intranasal instillation of

virulent *H. pertussis*. Mice in good health after seven days were counted as survivors.

Twenty mice were tested on each variable. Mice injected with suspension at weekly intervals showed 55% survivors, while those receiving extract showed 25%. Mice injected with suspension at three-week intervals showed 80% survivors, while 60% of those receiving extract survived. There were no survivors in the controls of either test.

The relative toxicity of the suspension and the extract was determined in mice. The M.L.D. of the suspension equalled 30,000 million organisms, while that of the extract equalled 280,000 million.

An injection of suspension (5,000 million) lowered the vitality of mice, but an equivalent dose of extract did not, as shown by the result of intranasal instillation of virulent *H. pertussis* at the intervals stated below:

PERIOD BETWEEN ANTIGENIC DOSE AND TEST	DEATHS IN 7 DAYS		
	Suspension	Topagen	Controls
	per cent	*per cent*	*per cent*
24 hours......	80	20	40
72 hours......	90	20	60
10 days.......	70	30	60
6 weeks......	40	15	100

ANTIGENIC STUDIES ON THE VARIOUS PHASES OF HEMOPHILUS PERTUSSIS. *Earl W. Flosdorf, T. F. Dozois and Anne C. Kimball*, Department of Bacteriology, University of Pennsylvania, School of Medicine, Philadelphia, Pa.

The existence of Leslie and Gardner's Phase I, III and IV has been confirmed. Each phase has a qualitatively separate and distinct agglutinogenic specificity

as determined by agglutination and agglutinin-absorption. They do not represent merely gradual changes in quantitative degree of reactivity. Within any given phase, however, strains do exhibit such quantitative differences.

No strains in the rarer Phase II have been found.

These data suggest: (1) Phase I organisms contain in their surfaces an antigen which is entirely lost upon transformation to Phase III; (2) the Phase III surface antigen is contained within the virulent strains but does not completely coat the Phase III organism; (3) very little Phase IV surface antigen is contained within the Phase I strains but it is found in Phase III, some of it on the surface; (4) no Phase III antigen remains on the surface of Phase IV organisms. A serum prepared against the filtered sonic extract of Phase I organisms confirms the fact that very little Phase IV agglutinogen exists within Phase I organisms.

Semi-quantitative agglutinin-absorption has shown that the low temperature ball mill extracts but 20% as much Phase I agglutinogen as the sonic method. Freezing and thawing liberates but negligible amounts.

THE PHYSIOLOGY OF LUMINESCENCE AND RESPIRATION OF LUMINOUS BACTERIA. *Frank H. Johnson*, Department of Biology, Princeton University, Princeton, New Jersey.

Although no luminescent extracts can as yet be obtained from bacteria, indirect evidence from many lines of study indicates that bacterial luminescence results from the oxidation of a substrate, *luciferin*, by a specific enzyme, *luciferase*. Quantitative studies of the intensity of luminescence and of respiration in relation to substrate, to osmotic pressure, and to inhibitors,

such as KCN, show that the intensities are not necessarily proportional. The CN-sensitive cytochrome oxidase can be greatly inhibited, while luminescence is only slightly, and indirectly, affected.

Studies of the "flash" (excess luminescence that lasts 2 or 3 seconds when oxygen is suddenly admitted to a suspension that is dim or dark because of oxygen-lack) indicate that, anaerobically, luciferin accumulates slightly, but quickly reaches an equilibrium which can be greatly altered by certain substrates. The kinetics of the bacterial flash, as well as that of *Cypridina* extracts, have been analyzed, on the basis of oscillograph records from a photocell-D.C. amplifier of a flow-method apparatus. All data indicate that at least the following reactions are concerned in luminescence:

1) $L + XH_2 \rightarrow LH_2 + X$
2) $LH_2 + A \rightarrow A \cdot LH_2$
3) $A \cdot LH_2 + \frac{1}{2} O_2 \rightarrow A \cdot LH_2 \cdot O$
4) $A \cdot LH_2O \rightarrow A' + L_1 + H_2O$
5) $A' \rightarrow A + h\mu$

In these equations, XH_2 represents a H-donator; LH_2, luciferin; and A, luciferase. The prime (') denotes an excited molecule.

ONE HUNDRED AND FORTY-SIXTH MEETING, PHILADELPHIA COUNTY MEDICAL SOCIETY BUILDING, MAY 28, 1940, PHILADELPHIA, PA.

SWINE INFLUENZA. *Joseph P. Scott*, University of Pennsylvania, School of Animal Pathology, Bristol, Pa.

Swine influenza virus is readily cultivated in the developing chick embryo. Egg passage virus was highly virulent for mice and pigs up to the 50th passage, 85th and later passages were nonvirulent for pigs and mice. Neutralization of virus by anti-serum was readily demonstrated in the developing chick embryo.

Mice infected with swine influenza virus developed lung lesions in three days, passage at 48-hour intervals increased the virulence of swine influenza virus strains.

Fourteen strains of swine influenza virus showed no differences in cross immunity and serum protection tests.

Pasteurella suiseptica cultures were non-pathogenic for pigs but activated swine influenza virus producing a non-contagious infection.

Untreated and formolized egg-passage virus produced a solid immunity in mice and caused some increase in resistance in pigs to experimental infection.

Swine influenza virus was isolated from the blood of pigs 10 to 34 days after experimental infection.

A carrier state is suggested by the development of immunity in pigs placed in contact with recovered animals and the spontaneous development of swine influenza in pigs three months after recovery from experimental infection.

THE RABIES SITUATION IN PENNSYLVANIA. *M. F. Barnes*, Chief, Division of Laboratories, Commonwealth of Pennsylvania, Department of Agriculture, Harrisburg, Pa.

The discussion contained an introductory statement concerning the early history of rabies in the United States followed by a presentation in tabulated and graphic chart form of the annual number of cases in animals during the last forty years accompanied by lantern slides; also the annual number of cases in dogs during the last fifteen years with their distribution in the 67 counties of the state. The total cases

occurring during each month of the year for the fifteen years and the average per month during this period were given. These data indicated that for the fifteen-year period the highest average number of cases occurred during the months of February, March and April, with March averaging the highest of the three months, while, during the so-called dog days of July and August, the monthly averages were the lowest, being lower for August than for July.

It is clearly shown that the total number of cases in the state fluctuated from year to year and as the result of enforcement measures in enzootic areas the enzootic centers shifted from one county, or group of counties to another. Cases were diagnosed in only two counties, Allegheny and Philadelphia, every year during the fifteen year period. No cases were diagnosed in twelve of the 67 counties, only one case in ten others, and 15 or more cases were diagnosed in only 23 counties, or an average of one or more cases per year. The report showed that the disease was readily eradicated from areas following quarantine, with other necessary enforcement measures and proper cooperation of the public.

BANG DISEASE. PRELIMINARY REPORT OF CALFHOOD VACCINATION. *M. F. Barnes*, Chief, Division of Laboratories, Commonwealth of Pennsylvania, Department of Agriculture, Harrisburg, Pa.

The discussion was given on the basis of a preliminary report representing a summary for a five-year period, terminating Jan. 1, 1940, of experimental calfhood vaccination in one herd and reference to surface information in the second herd, the results of which have not been summarized.

The experiments were begun to de-termine the merit under practical conditions in the average breeding herd of calf vaccination from the standpoints of safety and resistance to *Brucella* infection after the animals had reached maturity. The vaccine used was a living culture prepared from a strain of *Brucella abortus* (U. S. strain 19) of medium virulence.

Only two of the several groups into which the herd was divided were discussed. Group 2A consisted of 18 animals vaccinated between the ages of four and eight months and group 2B consisted of 17 control animals of similar ages. It was evident that the 2A group furnished a better record of performance than that of 2B or any of the other groups into which the herd was divided; more normal calves, lower average number of months to produce a full-term calf, a better breeding record, more milk, less sterility, no abortions, more satisfactory agglutination test results, and a record as good as that usually obtained with Bang-disease-free herds. In the control group, 2B, eleven of the 17 animals became positive to the agglutination test, nine of the animals aborted, eleven abortions occurred and a higher average number of months were required for a normal calf. The infections were from natural exposures in the infected herd. All groups of animals were intermingled with each other.

No definite conclusions were drawn because of the small number of animals involved, because surface information in the second herd indicates less favorable results, because of the possibility that resistance in the one herd was built up against infection with a single strain of *Brucella abortus*, and because of the wide-scale Federal experiments now nearing completion; but it is indicated that under limited conditions with proper definition calfhood vacci-

nation in certain infected herds can be used as a valuable adjunct in the control and elimination of Bang disease.

INCIDENCE OF CANINE LEPTOSPIROSIS AS DETERMINED BY THE AGGLUTINATION-LYSIS TEST OF SERA FROM DOGS IN PENNSYLVANIA. *Clara Raven and Kathryn Barnes*, Department of Bacteriology, Woman's Medical College of Pennsylvania, Philadelphia, Pa.

The agglutination-lysis test as recommended by Schüffner and Mochtar (Zentr. Bakt. Parasitenk., I., O., 101, 857, 1927) was found a satisfactory means for examining sera of dogs for evidence of a previous infection with leptospira. *Leptospira canicola* and *Leptospira icterohemorrhagiae* were the antigens used. The specificity of the two strains was determined by serologic studies and guinea pig inoculations.

Blood specimens from 105 dogs brought from rural portions of Pennsylvania were examined for serologic evidence of leptospirosis. In the course of this study it was found that 40 in 105 (38.1%) were seropositive. Specific reactions for *L. canicola* were three times as frequent as for *L. icterohemorrhagiae*. There was only a slightly greater incidence in the male (43.1% in 51) than in the female (39.1% in 46) animals. The *L. canicola* infection was much more prevalent in the female than in the male animals. Examination of the end-titers (up to 30,000) revealed that the number of higher titers was greater for *L. canicola* than for *L. icterohemorrhagiae*; however, paraspecific (cross-agglutination) reactions were more frequent for the group with high titers for the dog strain than for the classical strain, giving evidence for the greater specificity of the classical strain. A small proportion (4.7%) of the sera reacted equally to both strains—this could not be further tested in a non-clinical group of animals. In eleven instances (10.5%) for *L. canicola* and in seven instances (6.7%) for *L. icterohemorrhagiae* no cross agglutination was noted. This was suggested as evidence for the specific identity of the two stains. The findings in this study were found in close agreement with some of the work reported in the literature.

LEPTOSPIROSIS, A PUBLIC HEALTH HAZARD. *W. Paul Havens, Carl J. Bucher and Hobart A. Reimann*, Jefferson Hospital, Philadelphia, Pa.

In 1914 Inada and Ido described a spiral form of microorganism as the etiological agent of Weil's disease. Although the first proved cases of this syndrome occurring in Great Britain and in the United States of America were reported in the same year (1922) subsequent reports have listed 248 cases in the former and only 22 in this country. It is suggested that this discrepancy may be due to the facts that more interest is manifested abroad in this disease and that serodiagnostic and cultural methods, which are more commonly applied in Europe are frequently necessary for diagnosis, since jaundice occurs in less than 50% of the cases.

In July 1939, seven young men, bathing in a stream, possibly polluted by infected rats, contracted an acute infectious disease in varying grades of severity. Five were affected mildly and two were seriously sick with fever, jaundice and hematuria. One of the two severely ill patients died. Biologic studies of the fatal case and serologic evidence in the other severe case proved a diagnosis of *Leptospira icterohemorrhagiae*. Clinical and epidemiological evidence suggested a similar diagnosis in the mild cases although serologic proof was lacking.

WASHINGTON BRANCH

ONE HUNDRED AND FOURTEENTH MEETING, GEORGETOWN UNIVERSITY MEDICAL SCHOOL, APRIL 23, 1940

CONTRIBUTIONS TO BACTERIOLOGY BY LATIN AMERICANS. *Aristides A. Moll*, Panamerican Sanitary Bureau.

THE EFFECT OF CARCINOGENIC AGENTS ON SINGLE CELL ORGANISMS. *R. R. Spencer, and M. Melroy*, U. S. Public Health Service.

FIELD TESTS ON ZINSSER'S AND COX'S TYPHUS VACCINES IN HUNGARY AND ROUMANIA. Reported by *Dr. A. Eichhorn*, Director, Animal Disease Station, Beltsville, Md.

Zinsser, of Harvard University, and Cox, of the National Institute of Health, independently developed vaccines for prophylactic vaccination against typhus fever. Zinsser propagated the *Rickettsia* in tissue culture whereas Cox propagated it in developing chick embryo. Both vaccines proved highly potent in protection tests in guinea pigs. Because of the rarity of the disease on this continent, it was deemed advisable to test the vaccines on man in countries where the disease occurs in endemic or epidemic form. Hungary and Roumania offered such possibilities and both governments expressed their readiness for full cooperation in this effort.

Dr. Eichhorn was chosen by the National Institute of Health and by the Lederle Leboratories where the vaccine by the Zinsser method was prepared, to take sufficient quantities of the products to Hungary and Roumania and discuss a plan for the field tests. In Hungary, the Hygienic Institute, under the direction of Professor Johan, arranged for testing the vaccines in five villages in the sub-Carpathian region where typhus has been known to occur practically every season. The plan was to divide the entire population of the villages into three groups, one to receive the Cox vaccine, another the Zinsser vaccine, and the third to serve as controls. Those engaged in the organization and application of the tests also were to be vaccinated. The same plan was adopted by the Roumanian government for vaccination of approximately 10,000 persons in various villages of Bessarabia.

In the selection of individuals for these tests, all persons who have had typhus fever or react positively to the Weil-Felix Test, will be eliminated.

While this work is being undertaken somewhat late in the season, it was thought that because of the severity of the winter, the typhus season might be extended longer than usual. Unquestionably the vaccines have been placed in very reliable hands and the information to be obtained from the field application will furnish excellent data on the protective value of these products.

INDIANA BRANCH

PURDUE UNIVERSITY, MAY 24, 1940

THE STANDARDIZATION OF SNAKE VENOMS IN MICE IN TERMS OF HEMORRHAGIC UNITS. *A. E. Bolyn*, Purdue University.

The standardization of the venoms of *Crotalus atrox* and *Crotalus horridus* was effected by making serial dilutions of each of the venoms and injecting 0.25 ml. quantities, subcutaneously, over the peritoneum of white mice, and determining the smallest amount of venom that will cause a hemorrhage in

the peritoneum. The end-point, thus established, is referred to as a "Hemorrhagic Unit." The reactions are uniform and the titrations may be repeated with the same results, even if two mice are employed for each dose in a series.

PRODUCTION OF CITRIC ACID BY WILLIA ANOMALA. *H. C. Murray*, Purdue University.

Selected from several pellicle-forming wild yeasts, *Willia anomala* produced citric acid from four-per-cent glucose solutions. The citric acid was determined by Kogan's method. The maximum amounts of citric acid were approximately 80 mgm. per 100 ml. solution. This maximum occurred after five days of fermentation. Later the acid disappeared from solution. This may be of significance in the aerobic utilization of glucose by yeast.

THE INFLUENCE OF PHOSPHATIC AND POTASSIC FERTILIZERS ON NITRIFICATION. *B. E. Hahn, F. R. Olson, and J. L. Roberts*, Departments of Botany and Agronomy, Purdue University Agricultural Experiment Station, Lafayette, Indiana.

The rates of nitrification of ammonium sulfate added to soil samples from experimental plots, representing a variety of fertilizer treatments and two soil types, were determined. Each plot had received its respective fertilizer treatment at intervals for twenty years. Nitrification rates were determined five times on each soil during each of two growing seasons.

Although there were occasional slight differences indicating that phosphatic and potassic fertilizers may influence nitrification, these differences cannot be held significant without additional data. Seasonal variations in nitrification were observed. Nitrification in Bedford silt loam at Bedford, Indiana was most intensive in spring and late fall. There was a decline during summer and early fall. In Brookston silt loam at Lafayette, maximum nitrification occurred in late spring and early summer. There was a decrease during late summer and early fall. A slight fall rise occurred in 1938.

Laboratory experiments employing somewhat higher amounts of fertilizers than are commonly used in field practice indicated that phosphorus has little or no influence on the nitrifying capacity of these soils but that potassium chloride significantly reduces nitrification when applied in amounts greater than four hundred pounds per acre.

TRICHINOSIS AND ITS DIAGNOSIS. *H. E. Sandground*, Lilly Research Laboratories.

THE ELIMINATION OF CONTAMINATION. *James A. Reyniers*, Notre Dame.

MISSOURI VALLEY BRANCH

UNIVERSITY OF KANSAS, LAWRENCE, MAY 4, 1940

BETA-HEMOLYTIC LANCEFIELD GROUP C STREPTOCOCCUS IN BOVINE MASTITIS. *V. D. Foltz*, Kansas Agricultural Experiment Station, Manhattan, Kansas.

During the study of an epidemic of septic sore throat, while we were examining quarter samples of milk from the cows in a small farm dairy in an effort to determine the source of the trouble, one cow was discovered which was infected in one quarter with a Betahemolytic Lancefield group C streptococcus; the other three quarters

appeared to be normal. To our knowledge the treatment of this type of infection in the bovine with sulfanilamide has never been attempted and this animal afforded opportunity to study a number of points of value in mastitis control. Sulfanilamide was administered by mouth in sufficient dosage to keep the blood level of the drug near 6.0 mg. per cent for a period of 10 days.

The logarithmic average number of Beta-hemolytic streptococci per ml. milk from the infected quarter before, during and after treatment was 45,800, 17,500 and 55,000 respectively.

Leucocytes per ml. of milk averaged 11,500,000 before, 1,621,000 during and 2,282,000 after treatment. Composite samples of the three normal quarters gave the same general curve of fall and rise in leucocyte numbers but of a lower magnitude.

A microscopic examination of incubated samples of milk, by the Breed technique, was made to detect the presence of long-chained streptococci. Milk from the infected quarter was consistently positive for long-chained streptococci before treatment and until the blood level of sulfanilamide reached 6.5 mg. per cent. During the period of treatment, 10 days, this test was consistently negative, with one exception, until the blood level of the drug fell to zero; then the test again became positive. This is probably due to the bacteriostatic action of the sulfanilamide *in vitro*, a point which has led to erroneous conclusions by other workers.

The Hotis test was consistently negative throughout this experiment, indicating that this test was valueless in detecting this infection.

GROWTH OF SOME NON-SPORULATING ANAEROBIC BACTERIA IN SYNTHETIC MEDIA AND EXTRACTS FROM CORN SILK. *Robert A. West, Jr., Keith H. Lewis and Walter E. Militzer*, Departments of Bacteriology and Chemistry, University of Nebraska.

Only one strain among a group (varying from 18 to 30 in different experiments) of non-sporulating anaerobic bacteria of intestinal origin could be grown through four successive transfers in a mixture of inorganic salts, 17 amino acids, glucose, glycerol and sodium lactate.

The addition of crude and partially purified extracts of corn silk, as well as mixtures of known growth-promoting substances, allowed development of one-fourth to two-thirds of the strains.

The data thus far obtained indicate (1) diverse nutritional requirements within this group of non-sporulating anaerobes, (2) the importance of specific combinations of accessory growth factors and (3) the occurrence of physiologically active substances in corn silk which have not yet been obtained in pure form.

RESPIRATION STUDIES ON RESTING CELLS OF RHIZOBIUM MELILOTI. *John T. Kroulik and P. L. Gainey*, Kansas Agricultural Experiment Station.

Respiration studies on "resting cells" of "good" and "poor" strains of *Rhizobium meliloti* were carried out both by the Warburg and the methylene-blue reduction methods. Twenty-odd different substrates, including carbohydrates, alcohols and salts of organic acids, were tested. In general, results obtained by the two technics agreed. Among the more readily available substances tested were xylose, glucose, sodium succinate, sodium malate, levulose, and mannose. Raffinose and rhamnose were readily oxidized in the presence of methylene blue but not by the Warburg technic.

Citrate, acetate, malonate and hippurate were oxidized but little, if at all, by the methylene-blue method and with the exception of acetate, oxidation in the Warburg apparatus was relatively slow.

No consistent differences in the oxidizing abilities of highly efficient and inefficient strains or highly infective and noninfective strains were noted.

STREPTOCOCCI FROM RAW RETAIL MILK. *Edgar H. Beahm and Francis E. Colien,* Creighton University School of Medicine, Omaha, Nebr.

We have endeavored to group a large number of strains of hemolytic streptococci obtained from raw distributor milk samples of 45 Omaha dairies.

Milk samples from these dairies were plated in blood agar in dilutions of 1:100 and 1:1,000. After 48 hours of incubation at 37°C. the colonies of hemolytic streptococci were examined as to the type of hemolysis and picked into suitable culture media. Gram stains were made on each strain before they were inoculated into the carbohydrate media. All of the strains were inoculated into the following carbohydrate broths containing Andrade's indicator: glucose, lactose, sucrose, mannitol, maltose, salicin, glycerol, sorbitol and trehalose. They also were inoculated into litmus milk and sodium hippurate broth. Gram stains were again made on these strains after their inoculation into the differential media.

Each representative strain that was obtained by the biochemical methods referred to above is being typed serologically.

BACTERIA AS COLLOIDS. *T. M. Mc-Calla,* Kansas State College, Manhattan, Kansas.

Since each bacterium is a single cell and a unit of life, it affords an excellent tool to study the colloidal behavior of living organisms. Many of the changes that occur in a cell during growth, disinfection, staining and agglutination may be influenced by the colloidal properties of the bacterium. For example, in the utilization of carbohydrates by microorganisms some writers venture the explanation that the sugar molecule is attracted to the surface of the cell and broken down into smaller fragments which are transported into the organism.

In the staining reaction the present work supports the theory that when a dye molecule such as methylene blue is adsorbed by a bacterial cell it replaces some substance previously adsorbed by the organism. This was demonstrated by growing *Escherichia coli* in a nutrient agar medium containing 5 gm. of Mg^{++} as $MgSO_4$ per 1000 ml. The cells were washed free of excess salts and then a known concentration of methylene blue solution was added. When the methylene blue was adsorbed, Mg^{++} was released from the cell and was determined quantitatively. The data indicate that staining of a bacterial cell is an adsorption exchange process.

STUDIES ON NATURAL BACTERIOLYSINS. *Winston R. Miller,* U. of Kansas.

Natural bacteriolysins ("thermolabile" bactericidins) have been found in the fresh sera of many species of mammals. The bactericidal effect is a dual mechanism composed of an antibody-like factor and a complement-like factor. The work reported in this paper covers a general study of the presence of these natural bacteriolysins in the sera of mammals, aves, reptiles, and amphibians; and a specific study on the sera of cats and alligators with regard to specificity and the mechanism of reaction. The technic involves mix-

ing equal volumes of fresh serum with two dilutions of a suspension of bacteria, incubating three hours at 37°C., and plating-out the mixture in agar. By studying the colony counts on the plates, the bactericidal effect was determined. Quantitative estimations were made, but the results were mostly interpreted qualitatively. Laboratory stock strains of the following organisms were tested: *Salmonella schottmülleri, Salmonella enteritidis, Salmonella cholehaesuis, Eberthella typhosa, Shigella dysenteriae* (Shiga and Flexner), *Escherichia coli, Aerobacter aerogenes, Pasteurella avicida, Vibrio comma, Brucella abortus,* and *Proteus vulgaris* X19. The sera of dogs, rabbits, guinea pigs, man, rats, cats, chickens, turtles, alligators, snakes, and frogs were found to be bactericidal for some of the above organisms. Reptile sera were the most active, some of which had a definite bactericidal effect for all the organisms tested. The mechanism of the reaction was analysed using cat and alligator sera. A dual mechanism was found, composed of a "thermostable" amboceptor-like component and a "thermolabile" complement-like component. Absorption experiments showed the amboceptor to be at least partially specific. Very heavy suspensions were required for complete absorption. Considerable non-specific absorption appeared evident.

Bactericidal tests on the sera of very young animals showed definite bacteriolytic action with sera of bunnies as young as three days, and kittens and chicks as young as one day. The sera of 18-day chick embryos showed some bactericidal effect, but the embryonic fluids had no bactericidal effect.

It is hardly conceivable that specific bacteriolysins could be produced in reptiles and very young chicks as a result of clinical or sub-clinical infections with the above organisms. These appear to be truly *natural* antibodies.

USE OF AN ANTIGEN OF THE MAZZINI TYPE IN THE FLOCCULATION REACTION APPLIED TO CEREBROSPINAL FLUIDS. *L. O. Vose,* Missouri Valley Branch of the Society.

A flocculation technic for testing cerebrospinal fluid for evidence of syphilis consists of the use of an antigen of the Mazzini type with its sensitivity adjusted to a point believed too high for use in testing blood sera, but sufficiently low to avoid a level at which non-specific positive results might be expected with cerebrospinal fluid. After dilution the resulting emulsion is permitted to ripen for at least an hour and one drop (approximately 70 drops per ml.) is caused to fall on the clear unconcentrated spinal fluid in the bottom of each of two tubes. One of these tubes contains 0.1 ml. and the other 0.3 ml. of the fluid. These are agitated at about 240 oscillations per minute for a period of five minutes and are then read by use of a 2x magnifying glass and artificial light so adjusted to give maximum visibility of the flocculated particles. Definite flocculation in both or either tube is a positive, indefinite or very fine flocculation is doubtful and a silky appearance on being shaken characterizes a negative result.

A total of 738 spinal fluids were tested by this technic and by a sensitive complement fixation technic and the results compared, in which there was 93.9% of complete agreement, 3.0 of partial agreement and 3.1 of disagreement between the results of the two methods. It is concluded that a highly sensitive flocculation antigen adjusted on the basis of its behavior with spinal fluid, and for use with such fluids alone, may

be employed in a simple technic as a valuable adjunct to the complement fixation test in examining cerebrospinal fluids.

THE INCIDENCE OF SLOW-LACTOSE-FERMENTING ORGANISMS IN WATER SUPPLIES. *Ira G. Collins*, University of Kansas.

One hundred and twenty-five slow-lactose-fermenting organisms were isolated from 3,106 water samples received from October the 9th, 1939, to January the 9th, 1940, at the Kansas State Board of Health Water Laboratory. These organisms were obtained by inoculating 1 ml. of each sample into standard lactose broth and incubating at 37°C. The tubes were held for 14 days, unless fermentation appeared before that time. Eosin methylene-blue plates were used in the isolation of the organisms.

Out of the total samples received, 1,513 samples showed gas at some time during the 14 days incubation period. One hundred and twenty-five slow-lactose-fermenting organisms were isolated from these samples. Of these organisms, 28 were from raw waters, 5 from coagulation basins, 27 from filter effluents, 34 from finished waters, 21 from public school wells, and 10 from private wells.

A series of tests were run on the organisms, consisting of standard sugar mediums, (lactose, glucose, and sucrose), indole, methyl-red, Voges-Proskauer, sodium citrate, uric acid, gelatin, and hydrogen sulphide. From these 125 organisms, there were: 24 indole-positives, 48 methyl-red positives, 96 Voges-Proskauer positives, 109 that were sodium citrate positive, 104 that were uric acid positive, and 45 that liquefied gelatin.

In several cases slow-lactose-fermenting organisms were isolated from the same source at different times.

EASTERN MISSOURI BRANCH

SPRING MEETING, WASHINGTON UNIVERSITY SCHOOL OF MEDICINE,
ST. LOUIS, MAY 14, 1940

BACTERIOLOGICAL STANDARDS FOR PUBLIC WATER SUPPLIES AND THE TREATMENT REQUIRED. *H. O. Hartung*, St. Louis County Water Co.

Some investigators and authorities in public health are suggesting that the present accepted bacteriological criterion of a safe water supply (Treasury Standards—one coliform per 100 ml.) might not be a sufficient safeguard against all water-borne disease. These have indicated that mild and semi-mild intestinal disturbances, which become epidemic, could be water-borne, even though the water in question satisfied Treasury Standards. While epidemio-logical data do not support such an opinion, they argue that drinking water should be above mild suspicion, which they believe is possible only when the water is bacteria-free.

To produce a bacteria-free water is possible from an engineering viewpoint, but not without difficulties and considerable expense. Missouri River water may require as much as 20 p.p.m. of chlorine, if the chlorine contact period is about 24 hours. One to two p.p.m. of chlorine will kill coliform organisms, allowing water to meet present Treasury Standards.

If ammonia-chlorine treatment is

given the water, even 25 p.p.m. of chlorine will not completely sterilize the water. The pH of the water during treatment also has a tremendous influence on bacteria kill.

The water plant operator is very desirous of producing a water consistent with adequate public health safeguards, but at the same time is reluctant to subject his community to needless expense.

THE PROBLEM OF THE 48-HOUR GAS FORMER AND ITS SIGNIFICANCE FROM A PRACTICAL VIEWPOINT. *W. B. Schworm*, St. Louis Water Division.

The problem of the significance of the 48-hour gas former on lactose peptone broth is one which has engaged the attention of water bacteriologists for a number of years. These organisms are in the main spore-forming anaerobes, failing on Endo or EMB, highly resistant to chlorine, bromine, iodine and silver nitrate. The tolerance of the organisms for these bactericides was established by tests in the St. Louis Water Division Laboratories.

Present Treasury Department standards for drinking water for inter-state carriers does not include bacteria other than members of the colon group, as established by tests made according to Standard Methods of the American Public Health Association, but public health officials are inclined to look upon water supply with suspicion in outbreaks of gastro-enterititis. Whether this attitude is justified or not is debatable, as no clinical evidence has yet come to light establishing a connection between the chlorine-resistant 48-hour lactose fermenter and miscellaneous gastro-intestinal disturbances.

A program of determinative bacteriological collaboration of a number of Water Works Laboratories is suggested as a means of establishing this knowledge and ending the present dilemma.

PRACTICAL ASPECTS OF THE BACTERIOLOGICAL CONTROL OF PUBLIC WATER SUPPLIES. *W. Scott Johnson*, State Board of Health of Missorui.

Modern practices dictate that the safety of public water supplies shall depend upon four factors, (1) the condition of the raw water, (2) design and construction of the treatment plant, (3) operation and maintenance of the treatment plant, and (4) laboratory control, including water bacteriology. It has been long recognized that two factors are of vital importance in the practical use of bacteriological data in the control of public water supplies: (1) The analyses must be made in accordance with uniform standard procedure, therefore the development of the Standard Methods of Water Analysis approved by the American Public Health Association and the American Water Works Association, and (2) since it has proved impractical entirely to eliminate the coliform organism from treated water, a standard of allowable frequency of their occurrence, known as the Drinking Water Standard adopted by the United States Treasury Department.

Although from time to time Standard Methods and the Treasury Standard have been changed and improved, and future developments are desired, nevertheless, they have constituted a most useful, valuable and practical tool for those concerned with the safety of water for drinking purposes. Investigators have, from time to time, questioned the efficiency of these standards due to outbreaks of intestinal disturbances which had indications of being water-borne, but in most cases could not be definitely proven so. It has

been maintained among other criticisms that Standard Methods was (1) not sufficiently sensitive, (2) more time should be allowed for gas formation, (3) larger portions of water should be analized. Treasury standards are criticised as allowing too large percentages of 10 ml. quantities showing positive and due to the elimination of plate count.

LIFE ON NO MAN'S LAND. *Hans Bock*, Roosevelt High School, St. Louis, Missouri.

THE BIOCHEMICAL CLASSIFICATION OF YEAST STRAINS[1]

ALFRED S. SCHULTZ, LAWRENCE ATKIN, AND CHARLES N. FREY

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The classification of yeasts has been based to some degree on their ability to ferment certain sugars. This test is one of the well-established biochemical methods. Stelling-Dekker (1931) systematically examined the genus *Saccharomyces* and reorganized its classification unequivocally upon fermentation ability. However, this system, good though it be, is inadequate for the classification of the great number of yeast strains which, because of fermentative ability, must be called either *Saccharomyces cerevisiae* or *Saccharomyces carlsbergensis*. Designation of cultures as sub-species, varieties, strains and races is often based on individual characteristics which are evident in a particular process. The microbiologist is seldom able to identify exactly a fresh isolation or an unknown culture and must perforce carry it in his collection with a numerical designation. Although this inability may not be a great hindrance to industry, it is a serious problem for microbiology. The study of growth factors in particular has been greatly hindered by the lack of sharp differentiation between cultures.

As briefly reported elsewhere, Schultz, Atkin and Frey (1938–9) found a growth reaction which permits a sharp differentiation between otherwise closely related varieties of yeast. This difference appears in both the *S. cerevisiae* and *S. carlsbergensis* groups and does not appear to be related to the size or shape of cells so far as we have been able to determine.

[1] Read at the 41st General Meeting of the Society of American Bacteriologists, New Haven, Conn., December 28 to 30, 1939.

The test is based on the growth response to vitamin B_1 and B_6. A synthetic medium is prepared, which contains an adequate amount of the bios ingredients, as developed by W. L. Miller working with the yeast culture known as *S. cerevisiae* Toronto. By adequate bios we mean enough to give growth of *S. cerevisiae* Toronto equivalent to the crop obtained from 9° Blg. malt wort under the stated conditions of temperature, time, and seeding. The bios ingredients are Bios I (inositol); Bios IIA (β-alanine); and Bios IIB, a purified preparation readily made from a number of sources by carbon absorption and elution repeated thrice. We have employed cane molasses residues. It is necessary for the present work that the eluate be only substantially free from vitamins B_1 and B_6. The synthetic medium contains, in addition to glucose and inorganic nutrient salts, a buffer of potassium citrate and citric acid, designed to approximate the buffering capacity of malt wort. We have obtained a full crop with virtually all the yeasts tested, using $(NH_4)_2SO_4$ and β-alanine. Failure to consider all the known growth factors, or perhaps use of inadequate growth standards, may have led Nielsen and Hartelius (1939) to suggest that asparagine is necessary before β-alanine can act as a growth substance.

Twenty-nine milliliters of this sterile basic medium are placed in a 200 ml. erlenmeyer flask. The ammonium salt is sterilized separately to avoid reaction with glucose. One milliliter of a suspension containing one milligram of moist yeast is now added and the flask is shaken at 30°C. for 24 hours. Two milliliters of 10 per cent chloracetic acid are placed in the bottom of a Hopkins vaccine tube and then 1 to 5 ml. of the yeast suspension added. The tube is allowed to stand for 5 minutes before water is added to the 10 ml. mark and is then centrifuged at high speed for 5 minutes. The volume of yeast corresponding to 1 ml. of the yeast suspension multiplied by 10,000 gives the crop figures which are reported. It has been separately determined that 1 gram of moist yeast suspended in a volume of 30 ml. gives a crop, by this method of calculation, of 220. Thus, a crop figure in the neighborhood of 200 represents a thousandfold increase in yeast.

The yeast used to inoculate the growth tests is grown in an analogous manner on a malt wort of 9° Blg. prepared from Fleischmann's special diastatic dry Diamalt.   The pH is set at 5.0 with sulphuric acid and the whole autoclaved at 15 lbs. for 15 minutes, then filtered while hot and distributed in 30 ml. lots in 200 ml. erlenmeyer flasks.   These flasks, after intermittent sterilization, are inoculated from the agar slant with a platinum needle giving a fairly good-sized inoculum (roughly 1 to 2 cubic millimeters). All of the yeasts reported gave crops between 120 and 230, with the great majority around 200.   Other malt media or beer worts may be conveniently used.   A portion of the yeast suspension is prepared for use in the growth test by washing twice in sterile distilled water (in ordinary 15 ml. centrifuge tubes) and then diluting so that 1 ml. of centrifuged yeast is suspended in a liter of water, i.e., approximately one milligram of moist yeast per ml.

For the purposes of the growth tests to be described 10 gamma of thiamin is added to each 30 ml. of the basal medium and 10 gamma of each of thiamin and vitamin B_6 to another portion.

The cultures used were obtained from the American Type Culture collection, the Centraalbureau voor Schimmelcultures at Delft, and the Institut für Gärungsgewerbe at Berlin.   A number of cultures were identified in the American Type Culture collection as *Saccharomyces ellipsoideus,* but following Stelling-Dekker we have considered them as varieties of *S. cerevisiae* Hansen. The yeasts were cultured on malt agar.

RESULTS

Table 1 gives typical results on four *S. cerevisiae* cultures. Type A yeasts grow poorly on the basal medium and better with thiamin and still better when vitamin B_6 is added.   Types B and C yeast grow less well in the presence of thiamin, but B_6 overcomes the inhibition.   In our earlier publications we described only A and B type yeasts, but we have since found a few yeasts whose crops are depressed more than half by thiamin. It is felt that this difference is sufficient to justify a third type (type C).   On the other hand we have extended type B to cover those yeasts which show only slight inhibition by thiamin, or no inhibition at all.

We have thus far found ten strains (table 2) of yeast of type A, carried in the collections as varieties, strains or races of *S. cerevisiae* Hansen.

TABLE 1

Example of Saccharomyces cerevisiae Hansen types A, B and C

CULTURE	SOURCE	CROP VALUES			TYPE
		Basal medium	Plus thiamin	Plus thiamin and B_6	
Strain Delft I.	C. B. S.*	5	110	180	A
Strain Anamensis . ..	C. B. S.	230	200	220	B
Strain Fulmer no. 11 .	A. T. C. C. no. 4226	200	150	190	B
Strain Alpinus.	C. B. S.	150	35	200	C

* The following abbreviations are used in this and subsequent tables: A. T. C. C., American Type Culture Collection; C. B. S., Centraalbureau voor Schimmelcultures; I. f. G., Institut für Gärungsgewerbe; R. J. W., Roger J. Williams; W. L. M., W. Lash Miller.

TABLE 2

Saccharomyces cerevisiae Hansen type A

CULTURE	SOURCE	CROP VALUES		
		Basal medium	Plus thiamin	Plus thiamin and B_6
Var. ellipsoideus strain Delft II	C. B. S.	12	85	180
Strain Delft I.....	C. B. S.	5	110	180
Race XII	I. f. G.	5	180	190
Luft II	I. f. G.	15	170	190
Distillery yeast	A. T. C. C. no. 4111	90	170	205
Bakers yeast	A. T. C. C. no. 2335	5	60	130
Brewers yeast.	A. T. C. C. no. 2310	5	120	150
Distillery yeast...... ...	A. T. C. C. no. 4109	20	120	140
Wildiers yeast	R. J. W.	25	30	90
Distillers yeast....	A. T. C. C. no. 286	20	30	80

There is no reason for confusing any of these yeasts with types B and C. In spite of definite but reproducible differences among them, all show a great increase in growth in the presence of B_1

and B$_6$.   The double space separating groups of these yeasts may foreshadow a further subclassification of type A yeasts.   Such a

TABLE 3

Saccharomyces cerevisiae Hansen type B

CULTURE	SOURCE	CROP VALUES		
		Basal medium	Plus thiamin	Plus thiamin and B$_6$
Str. anamensis	C. B. S.	230	200	220
Str. batatae	C. B. S.	200	210	230
Nat'l. Coll. Type Cultures no. 467..	A. T. C. C. no. 2338	210	190	190
Tokay wine yeast..	A. T. C. C. no. 4108	180	170	180
"Magne" distillers . . .	A. T. C. C. no. 4132	200	190	200
Distillers	A. T. C. C. no. 4124	210	195	205
St. George wine	A. T. C. C. no. 4118	200	170	190
California wine	A. T. C. C. no. 4105	190	160	180
Wild yeast (American)... .	A. T. C. C. no. 4127	200	190	205
Race M.....................	I. f. G.	205	160	220
Spc. 152.	I. f. G.	210	160	220
Toronto strain	W. L. M.	205	170	200
Strain Fulmer no. 11.	A. T. C. C. no. 4226	200	150	190
Sulphite Yeast	A. T. C. C. no. 765	220	150	210
McDermott no. 74 .. .	A. T. C. C. no. 4100	200	130	190
Menes wine yeast .	A. T. C. C. no. 4117	200	150	190
German wine yeast . . .	A. T. C. C. no. 4129	200	130	180
Distillers yeast . .	A. T. C. C. no. 4125	180	130	200
Jordan wine yeast	A. T. C. C. no. 288	190	140	185
Laviero wine yeast ..	A. T. C. C. no. 4114	190	130	185
Hungarian beer yeast .	A. T. C. C. no. 764	190	130	190
French wine yeast	A. T. C. C. no. 4921	170	100	190
Distillers (Amer. grain) ..	A. T. C. C. no. 4110	140	120	205
French wine yeast	A. T. C. C. no. 4116	100	75	170
Ansmushausen wine. . .	A. T. C. C. no. 4113	100	50	120
Pribam collection	A. T. C. C. no. 2368	35	15	65

subclassification might readily be established even now but it is felt that it is not necessary at the present.   The fifth yeast has a higher-than-usual crop on the basal medium, whereas the last

five yeasts have lower crops on the medium containing both B_1 and B_6.

Of type B yeasts we have found 26 (table 3). Closer examination discloses that many are probably identical, but as mentioned before they have found their way into separate test tubes and no one would dare to mix them. As is apparent, these yeasts do not seem to require either thiamin or vitamin B_6. The first nine differ from the rest in so far as the depression of crop due to thiamin is at a minimum. The next thirteen are characterized by a somewhat greater drop in crop when thiamin alone is added.

The following three are similar to the first twenty-two but are characterized by a low crop on the basal medium. The last

TABLE 4

Types of Saccharomyces carlsbergensis

CULTURE	SOURCE	CROP VALUES			TYPE
		Basal medium	Plus thiamin	Plus thiamin and B_6	
Var. valdensis	C. B. S.	25	40	55	A
Strain Frohberg	C. B. S.	20	35	48	A
Chubut..	I. f. G.	220	180	220	B
Var. mandshuricus I. ...	C. B. S.	100	25	200	C
Var. polymorphous	C. B. S.	120	40	220	C
Kopenhagen.	I. f. G.	Nil	Nil	Nil	
A. T. C. C. no. 2345 ...	A. T. C. C.	Nil	Trace	Trace	

yeast is quite typical of B type yeasts, but all crops are low. As mentioned above, sub-types might readily be established on the basis of the differences shown, but we would like first to locate the deficiency made evident by the growth of such yeasts as the last one.

The type C yeast shown in table 1 is the only one which we have found under *S. cerevisiae.*

The results with seven strains of *S. carlsbergensis* are shown in table 4. The first two appear to belong to type A, although the low crops make them somewhat atypical. There is no question, however, that they differ from the other *S. carlsbergensis* strains. The third yeast is clearly a type B, and the next two are type C.

The last two *S. carlsbergensis* strains are in a class by themselves, inasmuch as the best medium is quite inadequate for them.

DISCUSSION AND CONCLUSIONS

The reaction of 37 strains of *S. cerevisiae* Hansen to the test described permits a definite and unequivocal subclassification without recourse to morphology. It is therefore suggested that cultures of *S. cerevisiae* be designated as *S. cerevisiae* Hansen Type A, etc. *S. carlsbergensis* might be treated in an analogous manner. Once the type has been determined, one may have recourse to morphological differences unless, as appears likely, further bios studies permit further biochemical classification. Recently Rainbow (1939) described a biochemical classification of yeasts based on bios tests. Unfortunately he was unable to repeat our observations with vitamin B_1 and B_6. His failure may have been due to the employment of a crude bios IIA preparation whereas we employ β-alanine. One may expect that other problems of classification in microbiology will be attacked and perhaps solved by the careful study of growth requirements and reactions.

SUMMARY

Examination of 44 yeast cultures described as strains, varieties or races of *Saccharomyces cerevisiae* and *Saccharomyces carlsbergensis* has shown that they may be further divided into:

Type A: yeasts which give a crop that is low on the basal medium, is increased by thiamin and further increased by vitamin B_6.

Type B: yeasts which give a crop that is high on the basal medium, is depressed by thiamin (not more than 50 per cent), and is normal in the presence of thiamin and B_6.

Type C: yeasts which give a crop that is depressed more than half by thiamin but which give a high crop on addition of both thiamin and B_6.

A few cultures are described which, because of low crops on all media, cannot be typed with certainty.

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THE TAXONOMY OF THE GENUS BACILLUS

I. Modes of Spore Germination

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A major problem of taxonomy is to discover and to learn how to apply successfully characteristics that are of significance in the differentiation of groups of organisms, and species. In general, morphological characters have been looked upon with great favor, so that classifications down to genera usually depend upon morphology. If a morphological character like spore germination can be applied to delimit groups or species, the taxonomy of the genus *Bacillus* will be thereby aided.

A review of the literature reveals considerable disagreement as to the correctness of using spore germination in taxonomy. The early investigators, especially Arthur Meyer's students (Gottheil, 1901; Neide, 1904), utilized this character as they considered it constant for any one species. Yet a perusal of species descriptions reveals a number of contradictory statements as to the type of germination that characterizes a given species. For example, in Marshall's "Microbiology" (1921) *Bacillus megatherium* is illustrated as germinating equatorially, while de Bary (1887), who originally described the species, claimed absorption of the spore coat. Gay's (1935) text records confusion in regard to the method which *Bacillus anthracis* exhibits. Grethe (1897) studied the question using five cultures of as many species and concluded that spore germination has no differentiating value. Ford *et al.* (1916) came to a similar conclusion. Current text books disagree; Salle (1939) believes germination to be generally constant while Henrici (1934) does not. Knaysi

347

(1938) in his recent review of the cytology of bacteria takes the sensible view that it may characterize some organisms and not others.

The present study, over a period of two and one-half years, has thrown some light on the subject. The length of time over which the observations were made allows an opinion as to the constancy of germination. The number of cultures of diverse origins studied permits some understanding of the taxonomic significance of spore germination.

CULTURES

A total of 105 cultures were studied, of which 77 were isolated by the author in 1936, 27 were received as named species and one as an unknown in 1937. They are listed under the species names assigned to them after a morphological, physiological, and serological (spores as antigen) study. The number of the culture in our collection is followed by a statement as to source. Those numbered with the letters C and S were supplied respectively by H. J. Conn of Geneva, New York, and M. H. Soule of the University of Michigan.

I. *Bacillus subtilis*

S8	*Bacillus subtilis*, Ford.
72	Soil.
58	Soil 40°C.[1]
43	Soil 45°C.
41	Soil 50°C.
78, 79	Horse manure 37°C.
60	Horse manure 45°C.
44, 45, 64, 65	Horse manure 50°C.
42	Air.
47	Creek.
61	Pasteurized whole milk.
70	Soil from a soy bean field 50°C.

[1] Temperature after source indicates the temperature of incubation at which isolations were made; otherwise the incubation temperature was 30–35°C.

II. *Bacillus agri*

13, 14, 15	Pasteurized skim milk 50°C.
21	Pasteurized skim milk 40°C.
66	Pasteurized skim milk 45°C.
80	Soil.

III. *Bacillus vulgatus*

C4	*Bacillus subtilis* (Marburg strain) by Conn.
C5	*Bacillus vulgatus* by Conn.
C8	*Bacillus arterrimus* by A. H. Robertson.
C11	*Bacillus leptosporus*, Klein. Pribram collection.
S1	*Bacillus mesentericus*, Kral. From Kral by Novy, 1890.
S9	*Bacillus subtilis*, no. 1 aus Strassenkchricht. Obtained from Grasberger, Wien, 1922.
S12	From eye infection. Isolated by Foster, Denver, 1937.
3	Large intestine of frog, *Rana palastrus*.
20	Pasteurized skim milk 40°C.
68, 69, 71	Cinders.

IV. *Bacillus mesentericus*

C7	*Bacillus mesentericus* by Conn.
S2	*Bacillus mesentericus* by Ford.
S10	*Bacillus subtilis* No. 4 from Preisz, Budapest, 1922.
18	Air.
23	Pasteurized skim milk 40°C.
51	Soil from a soy bean field.
54	Soil.
59	Horse manure 45°C.
62, 63	Soil from a soy bean field 45°C.

V. *Bacillus cereus*

C3	*Bacillus cereus* by Conn.
C12	*Bacillus petroselini* from Pribram collection.
C13	*Bacillus goniosporus* from Pribram collection.
C14	*Bacillus sessilis*, Klein from Pribram collection.
C16	*Bacillus subtilis*, Seitz, pathogen from Pribram collection.
S4	*Bacillus cereus* from Rettger 1922.
S6	*Bacillus subtilis*, Koch, from Germany by Novy, 1888.
S7	*Bacillus subtilis*, Kral, from Kral, 1890.

25, 34	Creek.
8, 12, 31, 52, 55	Soil.
16, 35, 37, 38, 39	Pasteurized skim milk.
24	Pasteurized skim milk, 40°C.
28	Air.
75	Cinders.
74	Moss.
56	Soil from a soy bean field.
1, 2, 4, 5, 6	Large intestine of frog, *Rana palastrus*.

VI. *Bacillus mycoides*

C2	*Bacillus mycoides* by Conn.
S5	*Bacillus mycoides*, University of Michigan collection.
36	Pasteurized skim milk.
40, 48, 50	Soil.
73	Moss.

VII. *Bacillus megatherium*

C1	*Bacillus megatherium* by Conn.
C10	*Bacillus niger* by A. H. Robertson (lacks black pigment).
S3	*Bacillus megatherium*, University of Michigan collection.
10	*Bacillus megatherium*, Cornell University collection.
19	Pasteurized skim milk 40°C.
26, 27	Creek.
7, 9, 32, 76, 77	Soil.

VIII. *Bacillus simplex*

| C6 | *Bacillus simplex* by Conn. |

IX. *Bacillus albolactis*

| C9 | *Bacillus albolactis* by Conn. |
| 17 | Pasteurized skim milk. |

X. Unidentified

| S11 | *Bacillus subtilis*, Str. Askanar, from Grumbach, Zurich, 1922. |

29	Horse manure 50°C.
46	Creek
49, 53	Soil.
57	Horse manure 37°C.

TECHNIQUE OF OBSERVATION

A slide culture method developed by Dr. Georges Knaysi of this laboratory was used to observe the method of bacterial spore germination. The technique is as follows:

1. A clean slide is sterilized in the bunsen flame and placed in a sterile petri dish.

2. No. 1 cover slips 22 mm. square are cut into 4 or 5 equal strips, flamed, and dipped into a sterile petri dish containing a shallow depth of sterile melted paraffin. The excess paraffin is allowed to run off.

3. Two of these strips are placed 15 to 16 mm. apart on the sterile slide parallel to each other and to the width edges of the slide. The melted paraffin solidifies and attaches the strips to the slide.

4. a. A clean no. 1 22 mm. square cover slip is flamed and placed on top of the strips. The warm cover slip melts some of the paraffin which resolidifies, and attaches the cover slip. A small quantity of a spore suspension is placed at the edge of the cover slip next to the space between the cover slip and slide. By capillarity the spores are drawn into the space; or,

   b. A drop of spore suspension is placed on a clean sterile no. 1 22 mm. square cover slip. The suspension is spread evenly and allowed to dry. The result is a thin layer of spores attached to the surface of the cover slip. The slip is warmed slightly and placed, spore layer facing the slide, on top of the strips.

5. By capillarity, agar at 45°C. is introduced into the space between cover slip and slide.

The above procedure gives a film of agar with bacterial endospores at its surface right beneath the cover slip. As the agar film is only slightly thicker than a no. 1 cover slip the microscope can be used to full advantage. The slide may be incubated and examined at intervals under the oil immersion objective, or placed in a stage incubator and germination examined continuously.

If any difficulty is experienced in initially focusing the spores it has been found advantageous to mark the cover slip on the same side as the spores with a china marking pencil. Then by focusing upon the china crayon mark the focus is on the same plane as the spores. By merely shifting the field the spores come under observation.

In these experiments, 3 per cent agar was found superior to 1.5 per cent agar as often the latter held sufficient water to allow Brownian movement, which makes observation more difficult. The nutrients were 0.2 per cent beef extract, 1 per cent peptone, and 0.05 per cent glucose.

Each culture was observed at least twice on two separate occasions and as many as five times for some cultures. The age of the spore was not found to affect the type of germination. Spores from cultures 1 to 16 days old were utilized, depending on the material at hand.

OBSERVATIONS

Before the work had proceeded far it was realized that often the type of germination was not a simple matter of absorption of the spore coat or of polar or equatorial germination. Important deviations were noted which are discussed under the headings of the particular type of germination. For a given culture, the particular type of germination proved constant. The observations convince one that germination is taxonomically significant, if we first properly catalogue the modes of germination, and then consider each mode separately.

The following is a separation of the modes of germination observed. All have been recorded on one occasion or another by various authors (de Bary, 1888; Grethe, 1887; Gottheil, 1901; Chester, 1904; Neide, 1904).

I. Spore germination by shedding of the spore coat. The characteristics of this method are three:

a. Spore does not expand greatly in volume previous to the germ cell breaking through the spore coat. The limit of volume increase of the spore may be considered to be twice its original volume.

b. Spore coat does not lose all of its refractive property previous to germination.

c. After the second division of the germ cell, giving a chain of three organisms, the original spore coat, remaining attached to the cells, is visible for a long time after germination. It is persistent.

A. Equatorial germination. 1. Spore coat does not split along the transverse axis. The germ cell forces its way through the coat at an angle approaching 90° to the longitudinal axis of the spore (figs. 1 to 4). The cell usually grows perpendicular to the longitudinal axis (fig. 4), but often the growth may continue parallel to that axis (fig. 5). So-called oblique germination occurs when the germ cell is at an angle of about 45° to the longitudinal axis when it first breaks through (fig. 6). In cultures showing this type of equatorial germination some spores could always be found with oblique germination. A few cultures that showed a greater number of spores with oblique germination also have many at an angle truly approaching 90°. Therefore it is to be concluded on the basis of the cultures studied that an organism may exhibit both types of germination mentioned in this paragraph, but not any other type. Example: *Bacillus subtilis.* Cultures 42, 43, and 44 show both this type and polar germination. Cultures 45 and 65 exhibit polar germination in the great majority of instances. However, these five cultures have many spherical, and barely oval shaped spores, for which it would be difficult to distinguish equatorial from polar germination.

2. Spore coat splits along the transverse axis (figs. 7 to 11). The growing germ cell forces its way through the wall and the spore coat splits along its transverse axis except for a narrow bit directly behind the growing cell. An analogous picture would be one of an egg split open and the two halve shells remaining attached in only one slight place. Example of such germination is *Bacillus vulgatus.*

B. Polar germination. The germ cell breaks through at an angle of 90° to the transverse axis of the spore (figs. 12 to 16). In the cultures designated *Bacillus agri* polar germination was recorded upon original isolation. During the intervening years,
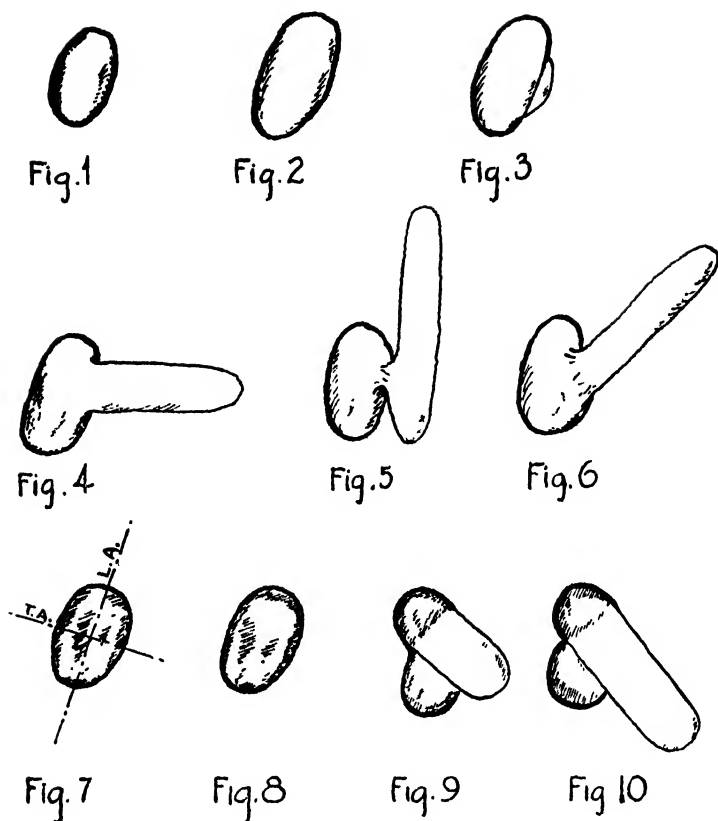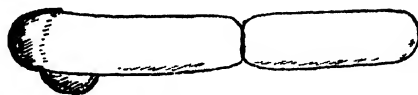
FIG. 11

FIGS. 1 TO 6. EQUATORIAL GERMINA-   FIGS. 7 TO 11. EQUATORIAL GERMINA-
TION WITHOUT SPLITTING ALONG   TION WITH SPLITTING ALONG
TRANSVERSE AXIS   TRANSVERSE AXIS

however, the picture changed slightly. The spores no longer remained refractive during germination. In this respect absorption of the spore coat is approached.

Fig.12   Fig.13   Fig.14
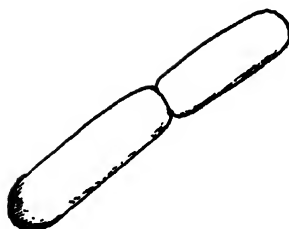
Fig.15   Fig.16

Fig.17   Fig.18   Fig.19
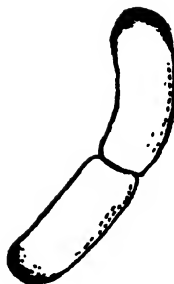
Fig.20   Fig.21

FIGS. 12 TO 16. POLAR GERMINATION   FIGS. 17 TO 21. COMMA SHAPED EXPANSION

C. Comma shaped expansion (figs. 17 to 21).   The growing germ cell splits the spore coat in half along the entire transverse axis.   The result is that two halves of the spore coat remain at either end of the growing cell which then shows two polar caps. As the cell grows, it becomes comma shaped in the majority of cases.   It acts as though a thin capsule still held the two halves of the spore coat together and the pressure exerted by the growing germ cell causes a buckling of the whole resulting in the comma shaped appearance.   This method of germination seems to be a development from the equatorial germination where the coat is split only in part along the transverse axis.   Further evidence for this view will be presented in a future paper where it will be shown that serologically both types of spores are related. It also seems to be a move in the direction of absorption of the spore coat, as many of the spores lose much of their refractiveness previous to germination.   Example: *Bacillus mesentericus*.

II. Spore germination by absorption of the spore coat (figs. 22 to 27)

The characteristics of this method are three.   a. The spore expands greatly during germination.   A tripling or greater increase of the original volume occurs (fig. 24).

b. The spore loses its characteristic refractiveness during germination so that it is difficult to say when the spore has disappeared and the germ cell appeared.

c. After the second division of the germ cell, even if a thin capsule originally remains, all traces of the spore coat are gone. Examples: *Bacillus cereus, Bacillus megatherium, Bacillus mycoides*.

Some strains germinating by absorption regularly show a thin capsule remaining about one end of the growing cell.   This would appear as a polar germination (fig. 28).   In other cases equatorially located capsules are seen (fig. 29).   Yet in all instances the spore is considered to germinate by absorption inasmuch as the three characteristics of the method are still adhered to.   Then again, the capsule, polar or equatorial, is not a constant feature. Many cells from the same culture will not show any.   Both polar and equatorially located capsules are found in any one culture

though one or the other may be preponderant. These so-called capsules, though probably originating as a part of the spore coat,

Fig. 22   Fig. 23   Fig. 24
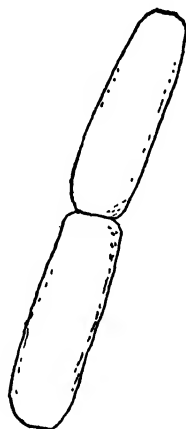
Fig. 25   Fig. 26   Fig. 27
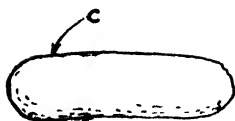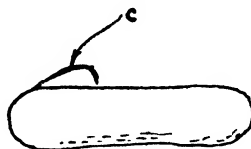
Fig. 28   Fig. 29

C - CAPSULE.

FIGS. 22 TO 29. SPORE GERMINATION BY ABSORPTION

are to be distinguished from the spore coat of the ungerminated spore in that their refractive property seems more nearly to

approach that of the cell wall of the germ cell than the refractive quality of the original spore coat. Therefore, germ cells with these capsules are not to be confused with those showing true polar and equatorial germination.

Of great significance is the fact that all of the organisms that Gottheil (1901) and Neide (1904) listed as having both polar and equatorial germination may be regarded as absorbing their coat, as they fulfill the three characteristics listed for this method. It may also be added that the greatest disagreement in the literature is to be found for the large-spored organisms (*Bacillus megatherium, Bacillus cereus, Bacillus mycoides*) whose method of germination is absorption as defined here.

The remaining observation to be recorded is that all the small-celled species studied showed coat-shedding types of germination, while all of the large-celled species showed absorption of the spore coat.

SUMMARY

When the methods of spore germination are clearly defined and separated they have taxonomic value. Spore germination of a given type is constant for any one culture and species.

The modes are summarized as:

I. Shedding of spore coat: small-celled species.
   A. Equatorial.
      Not splitting along the transverse axis: *Bacillus subtilis*.
      Splitting along the transverse axis: *Bacillus vulgatus*.
   B. Polar: *Bacillus agri*.
   C. Comma shaped expansion: *Bacillus mesentericus*.
II. Absorption of the spore coat: large-celled species.

The above outline does not claim to be complete. Rather it is hoped that it contains sufficient truth to be used as a convenient framework around which to build or from which to subtract according to the dictates of further investigation.

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PLATE 1

PHOTOMICROGRAPHS OF ACTIVELY GERMINATING SPORES OBSERVED IN COVERED
SLIDE CULTURES

FIGS. 1 TO 4. *Bacillus vulgatus.* Successive stages in equatorial germination
with splitting along the transverse axis. ×1100

FIG. 5. *Bacillus subtilis.* Early stages of equatorial germination without
splitting along the transverse axis. ×1100

FIG. 6. *Bacillus subtilis.* Late stages of equatorial germination. Remnants
of spore coat persist. ×1100

FIGS. 7 TO 10. *Bacillus megatherium.* Successive stages in germination by
absorption of spore coat. ×1100

(Carl Lamanna Taxonomy of Genus Bacillus)

FURTHER STUDIES OF THE ANTIBACTERIAL ACTIVITY OF SOME FLUORINATED AROMATIC MERCURIALS[1]

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INTRODUCTION

In a previous publication, Dunker and Grubb (1940) described their study of the antibacterial activity of several fluorinated aromatic mercurials having a single phenyl group. The preparation of these compounds was described by Dunker and Starkey (1939). This report presents the results of a similar study with two diphenyl fluorinated mercurials prepared by Heyman and Starkey (1940).

Only three previous studies have been made on the antiseptic or therapeutic value of diphenyl fluorine compounds. Thimm (1897) found that while p,p′-difluorodiphenyl *in vitro* did not kill *Streptococcus pyogenes, Staphylococcus aureus, Vibrio comma, Corynebacterium diphtheriae* or *Bacillus anthracis* in concentrations as high as 1:20, it did have a beneficial action on burns, wounds, suppurating ulcers and other infections of the epidermis. Valentiner (1899) reported that p,p′-difluorodiphenyl had a retarding effect on the growth of bacteria and that it diffused easily through animal membranes. He recommended a preparation of p,p′-difluorodiphenyl in lanolin for pertussis and for nonspecific oral and respiratory infections. For the treatment of wounds and burns, Thimm advised the use of an ointment containing difluorodiphenyl and fluoroxylol in lanolin. Thimm and Frankel (1927) recommended a mixture of p,p′-difluorodiphenyl

and p-fluorophenetole in lanolin for respiratory and rheumatic infections. In view of the claims made for p,p'-difluorodiphenyl and the results of Dunker and Grubb, it appeared of interest to study the antibacterial activity of the mercuri chloride derivatives of p,p'-difluorodiphenyl prepared by Heyman and Starkey.

EXPERIMENTAL

The chemical composition and some of the physical properties of the two compounds tested in this study are presented in table 1, together with those of 4-fluorophenyl mercuric chloride which

TABLE 1

Chemical composition and physical properties of the fluorinated aromatic mercurials tested for antibacterial activity

NAME	FORMULA	APPEARANCE	MELTING POINT	PER CENT Hg	
				Calculated	Found
4-fluorophenyl-mercuric chloride	HgCl—⟨ ⟩—F	Glistening plates	°C. 293–4	60.6	60.7 60.8
4,4'-difluoro-2-chloromercuri-diphenyl	F—⟨ ⟩—⟨ ⟩—F (HgCl)	Fluffy white needles	182	47.19	47.09 47.26
4,4'-difluoro-3-chloromercuri-diphenyl	F—⟨ ⟩—⟨ ⟩—F (HgCl)	White rosettes	199–200	47.19	47.06 47.09

was included in this study for the purposes of comparison—since it was found by Dunker and Grubb to be the most effective of the previously studied compounds.

The procedure followed in studying the antibacterial action of the above compounds was identical with that described by Dunker and Grubb. The mercurials were insoluble in water and hence 1:1000 stock suspensions were prepared in 0.5 per cent tragacanth. Serum dilutions were made without the use of the suspending agent.

The compounds were tested against the Food and Drug Administration strains of *Staphylococcus aureus* (no. 209) and *Eberthella typhosa* (Hopkins strain) in water and in undiluted sterile beef serum. All compounds were tested at the same time, using the same cultures of organisms to insure uniformity of conditions. The tests in water were carried out as follows: 100 ml. dilutions of the compounds, found by preliminary tests to fall within the proper ranges, were prepared from the stock 1:1000 suspensions. One ml. of a 24-hour broth culture of the test organism was inoculated into 100 ml. of the dilutions and after vigorous shaking 1 ml. samples were withdrawn after 1, 10 and 60 minutes exposure and plated in nutrient agar. The number of colonies developing on the plates were counted after 48 hours incubation at 37°C.

The compounds were also tested in undiluted serum in a similar manner with the exception that 0.1 ml. of the test organism was inoculated into 5 ml. of serum containing the various dilutions of the compounds.

<div align="center">RESULTS</div>

The results obtained when the compounds were tested in water dilution are indicated in table 2.

It is at once apparent that neither of the diphenyl compounds has as great an antibacterial activity against either of the test organisms as the 4-fluorophenyl mercuric chloride. The 4,4'-difluoro-3-chloromercuri-diphenyl is somewhat more active against both of the test organisms than the 4,4'-difluoro-2-chloromercuri-diphenyl compound, despite several inconsistencies to be expected in work of this type.

The results obtained when the compounds were tested in serum dilutions are shown in table 3.

In agreement with the findings of Dunker and Grubb, the presence of serum markedly reduces the antibacterial activity of the fluorinated aromatic mercurials studied here. Apparently these compounds are no exception to the general rule that mercurial antiseptics are considerably inactivated by the presence of serum (Nye 1937, Miller and Rose 1939). Here again, the

TABLE 2

Antibacterial activity of fluorinated aromatic mercurials in water dilutions

DILUTIONS	EXPOSURE	4-fluorophenyl-mercuric chloride		4,4'-difluoro-2-chloromercuri-diphenyl		4,4'-difluoro-3-chloromercuri-diphenyl	
		S. aureus	E. typhosa	S. aureus	E. typhosa	S. aureus	E. typhosa
	min.						
1:10,000	1	—	—	0	0	0	0
	10	—	—	0	0	0	0
	60	—	—	0	0	0	0
1:12,000	1	—	—	0	0	0	0
	10	—	—	0	0	0	0
	60	—	—	0	0	0	0
1:15,000	1	—	—	0	0	0	0
	10	—	—	0	0	0	0
	60	—	—	0	0	0	0
1:20,000	1	—	—	0	401	0	18
	10	—	—	0	13	0	0
	60	—	—	0	0	0	0
1:30,000	1	—	—	0	300	0	0
	10	—	—	0	7	0	0
	60	—	—	0	0	0	0
1:50,000	1	—	—	0	I	0	156
	10	—	—	0	450	0	6
	60	—	—	0	0	0	0
1:70,000	1	—	—	—	—	0	—
	10	—	—	—	—	0	—
	60	—	—	—	—	0	—
1:80,000	1	—	—	669	—	—	—
	10	—	—	I	—	—	—
	60	—	—	0	—	—	—
1:90,000	1	0	0	—	—	5	—
	10	0	0	—	—	0	—
	60	0	0	—	—	0	—

0, no growth in plate; I, innumerable colonies, plate not countable; —, test not run in dilution indicated.

6,400,000,000 *S. aureus* and 409,000,000 *E. typhosa* organisms respectively inoculated into 100 ml. of the aqueous dilutions.

TABLE 2—*Concluded*

DILUTIONS	EXPOSURE	4-fluorophenyl-mercuric chloride		4,4'-difluoro-2-chloromercuri-diphenyl		4,4'-difluoro-3-chloromercuri-diphenyl	
		S. aureus	E. typhosa	S. aureus	E. typhosa	S. aureus	E. typhosa
	min.						
1:100,000	1	0	0	7000	—	13	398
	10	0	0	I	—	0	8
	60	0	0	0	—	0	0
1:300,000	1	0	338	I	—	I	—
	10	0	0	I	—	30	—
	60	0	0	9000	—	454	—
1:500,000	1	0	315	I	—	—	I
	10	0	0	I	—	—	I
	60	0	0	I	—	—	I
1:700,000	1	54	563	—	—	—	—
	10	4	3	—	—	—	—
	60	0	0	—	—	—	—
1:1,000,000	1	10	I	—	—	—	I
	10	3	38	—	—	—	I
	60	0	4	—	—	—	465

4-fluorophenyl mercuric chloride is considerably more active than either of the diphenyl fluorinated mercurials; and the 4,4'-difluoro-3-chloromercuri-diphenyl is the more active of the two latter compounds.

DISCUSSION

The primary interest in carrying out the present series of experiments was to compare the antibacterial activity of the monophenyl and the diphenyl fluorinated mercurials. Our results indicate that the diphenyl compounds which we have tested are considerably less active than the monophenyl compounds, despite the fact that the former compounds contain twice the amount of fluorine.

In any study of chemotherapeutic or antiseptic compounds containing an aromatic nucleus it is of considerable interest to

TABLE 3

Antibacterial activity of fluorinated aromatic mercurials in serum dilutions

DILUTIONS	EXPOSURE	4-fluorophenyl-mercuric chloride		4,4'-difluoro-2-chloromercuri-diphenyl		4,4'-difluoro-3-chloromercuri-diphenyl	
		S. aureus	E. typhosa	S. aureus	E. typhosa	S. aureus	E. typhosa
	min.						
1:1,000	1	0	0	—	—	68	I
	10	0	0	—	—	117	I
	60	0	0	—	—	0	I
1:3,000	1	0	0	—	—	I	I
	10	0	0	—	—	I	I
	60	0	0	—	—	I	I
1:5,000	1	0	143	I	I	I	I
	10	0	158	I	I	I	I
	60	0	0	I	I	I	I
1:6,000	1	—	—	I	I	I	—
	10	—	—	I	I	I	—
	60	—	—	I	I	I	—
1:7,000	1	0	I	I	I	I	I
	10	0	I	I	I	I	I
	60	0	I	I	I	I	I
1:9,000	1	—	—	I	I	I	—
	10	—	—	I	I	I	—
	60	—	—	I	I	I	—
1:10,000	1	0	I	I	I	I	I
	10	0	I	I	I	I	I
	60	0	I	I	I	I	I
1:12,000	1	—	—	I	I	—	—
	10	—	—	I	I	—	—
	60	—	—	I	I	—	—

0, no growth in plate; I, innumerable colonies, plate not countable; —, test not run in dilution indicated.

595,000,000 *S. aureus* and 400,000,000 *E. typhosa* organisms respectively inoculated into 5 ml. of the serum dilutions.

TABLE 3—*Concluded*

DILUTIONS	EXPOSURE	4-fluorophenyl-mercuric chloride		4,4'-difluoro-2-chloromercuri-diphenyl		4,4'-difluoro-3-chloromercuri-diphenyl	
		S. aureus	*E. typhosa*	*S. aureus*	*E. typhosa*	*S. aureus*	*E. typhosa*
	min.						
1:15,000	1	—	—	I	I	—	—
	10	—	—	I	I	—	—
	60	—	—	I	I	—	—
1:20,000	1	—	I	—	—	I	I
	10	—	I	—	—	I	I
	60	—	I	—	—	I	I
1:30,000	1	5000	—	—	—	—	—
	10	4693	—	—	—	—	—
	60	4601	—	—	—	—	—
1:50,000	1	I	—	—	—	—	—
	10	I	—	—	—	—	—
	60	I	—	—	—	—	—
1:70,000	1	I	—	—	—	—	—
	10	I	—	—	—	—	—
	60	I	—	—	—	—	—

note the relation between activity and the relative position of the substituent groups attached to the ring. In the case of the fluorinated aromatic mercurials, we have been interested in the relation between antibacterial activity and the relative positions of the fluorine and mercuri chloride groups on the monophenyl and diphenyl nucleus. In table 4 an attempt has been made to summarize the data thus far obtained on this question.

In the monophenyl compounds studied by Dunker and Grubb it will be noted that there does not appear to be any consistent relationship between antibacterial activity and the position of the substituent groups, the relationship changing with different test organisms in different menstrua. On the other hand, with the two difluorodiphenyl compounds studied, the compound in which the mercuri chloride was ortho to the fluorine was con-

sistently more active than the compound in which the mercuric chloride was meta to the fluorine. No explanation for these findings can be offered at the present time with the small number of compounds that have been investigated, but they do serve to illustrate the great difficulty that is usually encountered when an attempt is made to correlate chemical structure with chemotherapeutic or antiseptic activity.

TABLE 4

Relation of antibacterial activity to relative positions of fluorine and mercuri chloride groups in fluorophenyl and difluorodiphenyl compounds

COMPOUNDS DILUTED IN	TEST ORGANISM	COMPOUND	POSITION OF FLUORINE IN RELATION TO HgCl GROUP		
			Ortho	Meta	Para
Water	*S. aureus*	Fluorophenyl	+++	+	++
		Difluorodiphenyl	+++	+	0
	E. typhosa	Fluorophenyl	+	++	+++
		Difluorodiphenyl	+++	+	0
Serum	*S. aureus*	Fluorophenyl	+	+++	++
		Difluorodiphenyl	+++	+	0
	E. typhosa	Fluorophenyl	+	++	+++
		Difluorodiphenyl	−	−	0

+++, most active; ++, intermediate activity; +, least active. (The designation of activity as +, ++ or +++ does not imply that one compound is one, two or three times as active as another compound, but simply the relative degrees of activity. A direct comparison of the relative activity of a fluorophenyl and a difluorodiphenyl compound cannot be made on the basis of this table.)

0, para isomer not possible with this compound; −, results did not demonstrate relative activity.

SUMMARY

1. A comparative study of the antibacterial activity of 4-fluorophenyl-mercuric chloride, 4,4'-difluoro-2-chloromercuri-diphenyl and 4,4'-difluoro-3-chloromercuri-diphenyl indicated that the monophenyl compound was considerably more active than either of the diphenyl compounds against *Staphylococcus aureus* and *Eberthella typhosa* in water and serum dilutions of the compounds.

2. The 4,4'-difluoro-3-chloromercuri-diphenyl compound was more active in water dilutions against *E. typhosa* and *S. aureus;* and serum dilutions against *S. aureus* than the 4,4'-difluoro-2-chloromercuri-diphenyl. The results obtained with serum dilutions of the compounds against *E. typhosa* did not permit any conclusions to be drawn regarding the relative activity of the compounds.

3. An analysis of the relation between antibacterial activity and the relative positions of the mercuri chloride and fluorine groups on the monophenyl and diphenyl compounds indicated that while no general conclusion could be drawn concerning the monophenyl compounds, the two diphenyl compounds consistently displayed greater activity when the substituent groups were in the ortho rather than the meta position to each other.

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THE VACCINATION OF MICE WITH A ROUGH STRAIN OF STREPTOCOCCUS[1]

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Spicer and Bloom (1938) have reported results which they obtained in inducing non-type-specific immunity in mice against hemolytic streptococci with a vaccine prepared from a rough variant of a strain of streptococcus (B_3R). They stated that vaccine made from the rough variant gave better protection than that made from the smooth parent strain. Working with a vaccine prepared from this strain, Spicer and Bloom noted that when the vaccine was detoxified with the aid of alcohol and ether it could be injected into mice in large doses without causing ill effects. A short course of injections was reported to give a high degree of non-type-specific immunity with the mice surviving injections of 10,000 minimal lethal doses of a virulent heterologous strain of streptococcus.

We were very much interested in the further development of the work started by these investigators as we fully agreed with their statement that there is an urgent need for non-type-specific vaccine and serum against streptococci, if such could be prepared. For this reason we undertook the study reported below.

EXPERIMENTAL

Preparation of vaccine

In all cases, except as otherwise noted, the strain used for vaccine was the B_3R strain of Spicer and Bloom. The present study was done two years after Dr. Spicer's original work, and

the variant had been maintained by transplant since that time. On blood plates this strain gave colonies of from 2 to 4 mm. in diameter surrounded by a wide zone of well defined β-hemolysis. It had a coarsely granular surface and an uneven edge; however, the colony appearance was not that of a typical rough. The organism grew as a sediment in broth and upon shaking the sediment came up as a "cottony" mass. In stained smears no pleomorphism was shown as is the case with typical roughs. Our conclusion was that while this strain would fall into Dawson's "rough" colony form (Dawson, Hobby and Olmstead, 1938), it was not typical of such strains in every respect.

The method of preparing the vaccine was as close a duplication of the method of Spicer and Bloom as was possible. However, it will be repeated here. The strain was kept on blood-agar slants and when desired for use, was streaked on a blood-agar plate, incubated overnight, and a typical colony fished into blood broth the next morning. After six hours incubation in the blood broth, a transfer was made into plain infusion broth. This was incubated for 18 hours and after incubation was used to seed Blake bottles of hormone agar. Each bottle contained 100 ml. of agar and was seeded with 8 ml. of broth culture. The bottles were incubated for 18 hours and the growth washed from the surface with sterile saline.

The bacterial suspension was centrifuged, washed with absolute alcohol and incubated in a 50°C. water bath for 30 minutes. After incubation, the cells were centrifuged down, the alcohol discarded, and the sediment suspended in absolute ether (10 times the bulk of the sediment). They were stirred in the ether with the aid of an electrical stirrer for 30 minutes. The ether was removed by centrifugation and the sediment dried. To the dry cells formalinized saline was added (0.1 per cent formalin), and the vaccine standardized to contain nine billion organisms per ml.

Immunizing and testing

White mice of 20 to 23 grams in weight were used for the immunization. Each mouse received three intraperitoneal injections on alternate days; the first two injections consisting of

0.5 ml. of the vaccine, and the third 1.0 ml. of the vaccine. The mice were rested for seven or eight days and then tested. The mice injected with infusion broth only were treated according

TABLE 1

TEST ORGANISM	DOSAGE OF 18 HOUR CULTURE	NUMBER OF MICE	PERCENTAGE SURVIVING	AVERAGE TIME INTERVAL BEFORE DEATH
(1) Controls, no vaccination				
	ml.		*per cent*	*hours*
1685M	10^{-6}	16	0	26.8
1685M	10^{-7}	31	10	30.8
Pneumococcus type 1 mucoid	10^{-4}	8	0	28.0
Pneumococcus type 1 mucoid	10^{-6}	20	0	35.7
(2) Mice vaccinated with B_3R				
1685M	10^{-4}	38	5.3	35.8
1685M	10^{-5}	39	20.5	40.6
1685M	10^{-6}	67	39.0	45.3
1685M	10^{-7}	29	44.5	39.0
Pneumococcus type 1 mucoid	10^{-4}	8	0	28.0
Pneumococcus type 1 mucoid	10^{-6}	20	0	35.7
(3) Mice vaccinated with infusion broth only				
1685M	10^{-4}	6	0	24.0
1685M	10^{-5}	6	16	38.4
1685M	10^{-6}	6	16	43.2
1685M	10^{-7}	5	20	51.0
(4) Mice vaccinated with a heterologous mucoid strain of streptococcus (1048M, Type 6)				
1685M	10^{-4}	6	16	43.2
1685M	10^{-5}	6	50	36 0
1685M	10^{-6}	6	0	49.6
1685M	10^{-7}	6	16	43.2

to the same schedule; 1 ml. of broth being the dosage used at each injection. A mouse-virulent erysipelas strain of streptococcus (1685M, group A, type 1) was used in most cases for

testing the degree of active immunity established. Other strains were used as designated in the protocols. The mice living 96 hours after the injection of streptococci were recorded as surviving. The results of the experiments at the University of Pennsylvania are summarized in table 1.

TABLE 2

TEST ORGANISM	DOSAGE OF 18 HOUR CULTURE	NUMBER OF MICE	PERCENTAGE SURVIVING	AVERAGE TIME INTERVAL BEFORE DEATH
(1) Mice vaccinated with B₃R and tested with 1685M				
	ml.		*per cent*	*hours*
1685M	10^{-4}	6	0	36.0
1685M	10^{-5}	6	0	36.0
1685M	10^{-6}	6	0	43.3
1685M	10^{-7}	6	33	52.0
1685M	10^{-8}	6	66	72.0
(2) Controls, no vaccination				
1685M	10^{-6}	3	0	32.0
1685M	10^{-7}	4	0	54.0
1685M	10^{-8}	3	33	42.0
(3) Mice vaccinated with B₃R and tested with S206				
S206	10^{-3}	6	16	36.0
S206	10^{-4}	6	0	36.0
S206	10^{-5}	6	50	72.0
S206	10^{-6}	6	84	72.0
S206	10^{-7}	6	84	96.0
(4) Controls, no vaccination				
S206	10^{-6}	3	0	32.0
S206	10^{-7}	3	0	56.0
S206	10^{-8}	3	33	36.0

Through the kindness of Dr. Ralph Muckenfuss and Dr. Sophie Spicer this work was repeated in part at the laboratories of the New York City Department of Health, under the direction of Dr. Spicer. The results obtained are summarized in table 2. In these experiments one of Dr. Spicer's strains of streptococci (S206 septicemia) was used in addition to 1685M for testing.

The results summarized in these two tables show that it was possible to obtain some protection by injecting, intraperitoneally,

large doses of a vaccine made from a rough strain of streptococcus; there was considerable variation, however, and the protection seemed to be better against Spicer's S206 strain than against the 1685M strain.   A similar vaccine made with a heterologous mucoid strain (1048M) likewise gave some protection against 1685M.   It was also noted that injections of infusion broth showed some protective activity.   The B$_s$R vaccine did not give a non-specific protection against a mucoid pneumococcus. While the protection obtained in this study was not as striking as that reported by Spicer, there was some indication of protection.

TABLE 3

Examination of peritoneal exudates from mice twenty-four hours after infection

MICE IMMUNIZED WITH	NUMBER OF MICE	TEST DOSE OF 1685M	CELL COUNT			STREPTOCOCCI SEEN IN SMEAR
			Poly-morpho-nuclears	Mono-cytes	Lympho-cytes	
		ml.	*per cent*	*per cent*	*per cent*	
Non-immunized	3	10^{-7}	90	2	8	Numerous
Non-immunized	1	10^{-8}	77	15	8	Numerous
Infusion broth.......	3	10^{-7}	70	14	16	Few
Infusion broth	1	10^{-7}	73	24	4	Numerous
Infusion broth	1	10^{-7}	32	60	8	None
Infusion broth	1	10^{-7}	47	45	8	None
B$_s$R vaccine..	3	10^{-7}	43	20	37	None
B$_s$R vaccine....	1	10^{-8}	50	45	5	None
B$_s$R vaccine....	1	10^{-7}	47	28	25	Few

It was also noted that the vaccinated mice (both B$_s$R and infusion broth) that did succumb following the test dose of culture, died, on the average, about ten hours later than unvaccinated controls.

A study of the mechanism of this protection was then made. Some mice which had undergone a course of injections with B$_s$R vaccine were bled and their sera tested for agglutinins against B$_s$R, type 1 and type 6 streptococci.   Negative results were obtained.   Three rabbits were given a series of injections of the B$_s$R vaccine and the serum tested for agglutinins; only a weak response was obtained.

The next step consisted of examining microscopically peritoneal exudates from immunized and normal mice after they had been injected with the test dose of organisms. (The smears were stained with Wright's stain). The results are given in table 3.

The main difference observed was the increase in monocytic response in the mice injected with B_3R vaccine and with infusion broth twenty-four hours after infection. This may be the factor responsible for the increased resistance of the immunized mice, as the smears showing an increase in the number of monocytes also showed few or no streptococci. Gay, Clark and Linton (1926) have shown that monocytes play an important rôle in local resistance to streptococci. Webb (1939), working in their laboratory, has recently extended these observations to *Pasteurella lepiseptica* infection in rabbits. He came to the conclusion that "Mononuclear accumulations . . . were definitely correlated with enhanced resistance of rabbits to intrapleural infection."

SUMMARY

This study shows that there does seem to be some protection against Group A hemolytic streptococci produced in mice when they are injected, intraperitoneally, with large doses of a specially prepared vaccine made from a rough strain of streptococcus. Some protection is also afforded by injections of infusion broth. This protection is non-type-specific and seems to be associated with the increased mobilization of monocytes in the peritoneal cavity rather than to any specific immune mechanism.

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ON THE MICROÖRGANISMS OF THE LOWER LIMITS OF THE BIOSPHERE[1]

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Studies on the underground waters (during oil drilling) from considerable depths in the region of Baku have shown the presence in them of sulfate-reducing, thiosulfate-oxidizing, denitrifying and other bacteria, indicating biogenic transformations of sulfur, formation of H_2S and of N_2 (Ushinsky, 1926; Ginsburg-Karagitscheva, 1933; Maliantz, Reinfeld). The first findings of bacteria at great depths naturally evoked doubts concerning the accuracy of the observations, since bacteria might penetrate from higher strata, but further studies have corroborated the evidence of the occurrence of microörganisms down to a depth of 2000 meters. If doubts could remain concerning the wide spread of bacteria reducing sulfates and nitrates, the finding of considerable numbers of purple sulfur bacteria in waters spouting from depths of 1400 to 1700 meters, (artesian wells) gave new and significant evidence concerning the extension of the depth of the biosphere.

The geological picture of the region, in which the exudance of "pink water" is observed from many drillings does not completely eliminate the possibility of the penetration of large numbers of purple bacteria from the surface. However, the evidence that they occur primarily at great depths is more conclusive.

Volodin first noted the appearance of "pink water" at a depth of 1500 meters. The well at first gushed oil with a slight admixture of water (2 to 15 per cent), and later the amount of water rose to 100 per cent. With the increase in water content of the oil, the pink color became less pronounced.

[1] Translated by Dr. M. Doudoroff.

The following considerations speak against any but a deep origin of the pink water:

One well opened December 19, 1933, gave 1500–2000 tons[s] daily. Pink water accompanied the oil at first in about 15 per cent ratio, later decreasing to 2 to 3 per cent, and was bright pink in color. After 3 months, the proportion of water rose to 60 per cent with the same bright color, while after 4 months, it reached 100 per cent, with a quantity up to 400 tons daily.

In other wells (more than 20), the general picture was the same. The water from the drillings has a salinity of 4.4 to 5.1° Baumé with a greater amount of sulfates than other waters from the same depths. The maximum H_2S concentration was 0.037 gram per liter. It is unquestionable that the "pink water" with the purple bacteria came from deep accumulations of water with the oil in the "dome." It is quite possible that the bacteria developed at the interface of water and oil, forming a layer, as can be seen on the surface of the earth in basins with oil. Beyond the contour of the oil-bearing layer there are waters uncolored with H_2S, which penetrate the curved part of the contour (Malishek).

The first analyses of "pink water" by Maliantz and Reinfeld showed the presence of large numbers of *Chromatium* of various sizes and *Thiospirillum*, leaving no doubts that the color was due to sulfur purple bacteria.

To study these organisms, I used various media and found that they grow beautifully in organic media. The cherry-red color appears on the 4th to 5th day. Best results were obtained in anaerobic vessels, although some forms developed in the presence of air. By their appearance (in culture media) the bacteria belonged to the *Athiorhodaceae* not containing sulfur and to the *Thiorhodaceae* with sulfur. Many *Chromatium* of various sizes could be found, as well as forms resembling *Rhabdochromatium*.

Judging by the spectrum of the extracts of cultures, the cells contain both bacteriochlorin and bacteriopurpurin, the latter

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[s] "t" in original; assumed to mean "tons."

showing some modification differentiating it from both α and β bacteriopurpurins.   It must be pointed out that in the darkness the bacteria showed almost no development, but several observations give evidence that the addition of crude oil to the water acts somewhat beneficially, and some *Athiorhodaceae* developed in the dark in the presence of oil.

The finding in oil of various microörganisms is one of the indications of its biogenic origin.   The presence of purple sulfur bacteria may be due to the internment of marine algae with the accompanying microörganisms.   If this is true, they must be considered relic organisms.   Upon reaching the surface of the earth they develop under a layer of oil.   This picture may be seen in many places in the vicinity of oil wells.   Further studies must show the validity of the supposition that they are relic organisms.

The fact that along with the purple bacteria, underground waters contain not only anaerobic organisms, but also aerobes, such as the thiosulfate oxidizers, may be explained by the formation of oxygen at great depth in the splitting of water by x radiations of radium and mesothorium (according to Vernadsky, 1927) since the water has a radium-mesothorium character.

Thus we may conclude that the limits of the biosphere can be extended to 2000 meters.

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THE BACTERIOSTATIC ACTION OF SULFANILAMIDE UPON HEMOLYTIC AND NON-HEMOLYTIC ENTEROCOCCI

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During the last few years, it has been established beyond doubt that sulfanilamide and allied compounds such as sulfapyridine, prontosil and others, are effective both *in vitro* and *in vivo* against a large variety of quite unrelated microörganisms. Thus, sulfanilamide has a curative action in infections due to beta-hemolytic streptococci, Lancefield Group A, meningococci, *Clostridium welchii*, and even plasmodia such as *Plasmodium knowlesi*. Certain microörganisms are considered to be rather resistant to the bacteriostatic and bactericidal action of these chemotherapeutic substances. It was shown by Helmholz and Osterberg (1937); Kenny, Johnston, and Haebler (1937); Bliss and Long (1937); Long and Bliss (1938); and Neter (1938–1939a) that under certain experimental conditions sulfanilamide lacked definite growth-inhibitory action toward members of the enterococcus group, including Lancefield Group D hemolytic streptococci and non-hemolytic enterococci. Recently, however, Bliss and Long (1939) reported that sulfanilamide may definitely delay the growth of enterococci, provided that high concentrations of the drug and small inocula are used. Subsequently, it was shown (Neter, 1939b, 1940) that sulfanilamide in a concentration of 1 per cent may delay or completely inhibit the growth of hemolytic enterococci in a culture medium of high (6.5 to 7 per cent) sodium chloride content. Furthermore, it was found that the bacteriostatic action of 1 per cent sulfanilamide toward

both hemolytic and non-hemolytic enterococci is greatly enhanced at approximately 43°C. in comparison to that obtained at 37°C. In the following communication a more detailed quantitive study on the bacteriostatic action of sulfanilamide toward members of the enterococcus group is presented.

MATERIAL AND METHODS

Eight strains belonging to the enterococcus group were employed. Two of them were obtained through the courtesy of Dr. J. M. Sherman, Ithaca, New York. Three strains (4080, 4081, and 7080) were secured from the American Type Culture Collection. The remaining strains were isolated in this laboratory from human sources.

The basic culture medium used was a phenol-red broth base (Difco) containing 1 per cent tryptose, 0.5 per cent sodium chloride, 0.1 per cent dipotassium phosphate and phenol red as indicator. The respective amounts of sodium chloride (Merck) and maltose, C.P. (Pfanstiehl) were added to this broth base. As sulfanilamide, prontylin (repurified for injection, Winthrop) was employed. The final culture medium was autoclaved at 15 pounds pressure for 15 minutes. It was used only if there was no noticeable change in the pH following this procedure.

The number of viable microörganisms was determined by means of poured blood-agar plates. One milliliter of citrated normal human blood, 10 milliliters of brain veal agar (Difco) and 0.2 milliliter of the specimen to be examined, were used. The control and sulfanilamide broth cultures were tested in the following dilutions: 1:1; 1:100; 1:10,000; 1:1,000,000; and 1:100,000,000. The dilutions were made in broth rather than in physiological saline solution, in order to avoid the deleterious effect of the latter on the microörganisms. The poured blood-agar plates were incubated at 37°C. for at least 72 hours, until the maximum number of colonies had developed.

RESULTS

In the first experiment, the bacteriostatic action of sulfanilamide in a concentration of 1 per cent upon a strain of hemolytic enterococcus in sodium chloride broth was determined. Different

dilutions (1:1; 1:50; and 1:2,500) of an 18-hour broth culture in a volume of 0.1 milliliter were used for inoculation of both the control broth and the sulfanilamide broth.   In this particular experiment, the sodium chloride content of the control broth was made up to 6.5 per cent, while that of the 1 per cent sulfanilamide broth was only 5.5 per cent.   This order of experiment was chosen in order to overcompensate for increased osmotic pressure of the sulfanilamide broth, thus rendering the conditions in regard to osmotic pressure even less favorable in the control broth than those prevailing in the sulfanilamide broth.   The tubes were

TABLE 1

Bacteriostatic action of sulfanilamide (1 per cent) upon hemolytic enterococcus

Control broth: 6.5 per cent NaCl-maltose-phenol red broth. Sulf. broth: 5.5 per cent NaCl-1 per cent sulf.-maltose-phenol red broth.

HOURS OF INCU-BATION AT 37°C.	INOCULUM (18 HOUR BROTH CULTURE)					
	A, 0.1 cc.		B, 1:50 0.1 cc.		C, 1:2,500 0.1 cc.	
	Control broth	Sulf. broth	Control broth	Sulf. broth	Control broth	Sulf. broth
18	+	+	−	−	−	−
24	++	++	−	−	−	−
48	+++	+++	−	−	−	−
72	+++	+++	++	−	−	−
96	+++	+++	+++	−	−	−
9 days	++	++	+++	−	+	−

−, no visible growth; + to +++, various degrees of visible growth.

incubated at 37°C. for 9 days.   Visible growth was noted at various intervals.   The results of this experiment are presented in table 1.

It may be seen from table 1 that sulfanilamide in a concentration of 1 per cent failed to inhibit visible growth of the hemolytic enterococcus, when undiluted broth culture was used for inoculation of the test culture medium.   With smaller inocula, however, a marked bacteriostatic effect could be demonstrated.   Essentially the same results were obtained in repeated experiments with both hemolytic and non-hemolytic enterococci.   These observations on the relationship between the degree of bacteriostatic effectiveness of sulfanilamide and the number of microörganisms

used for inoculation, parallel those obtained with hemolytic streptococci and other bacteria.

In the next series of experiments, a quantitive analysis was made of the influence of sulfanilamide on the growth and death rate of hemolytic enterococci *in vitro*. As culture media, 6.5 per cent sodium chloride, $\frac{1}{4}$ per cent maltose phenol-red broth, with and without 1 per cent sulfanilamide, were used. Various sizes

BACTERIOSTATIC ACTION OF
SULFANILAMIDE (1%)
ON
HEMOLYTIC ENTEROCOCCUS
IN
6.5% NaCl–¼% MALTOSE BROTH
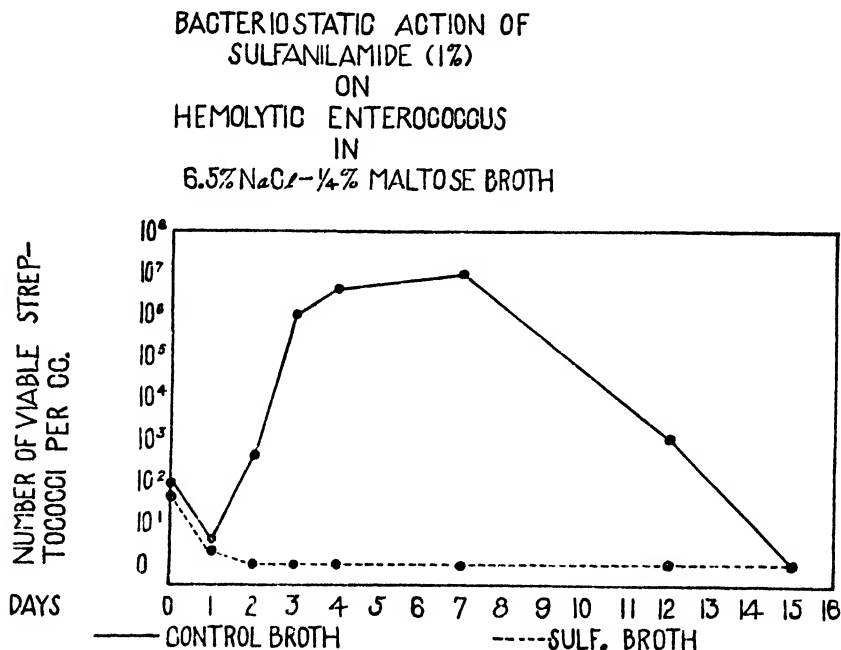
FIG. 1

of inocula of a strain of hemolytic enterococcus were tested. The results of 3 representative experiments are given in figures 1, 2, and 3.

It may be seen from figure 1 that, with a relatively small inoculum (80 to 100 viable microörganisms per milliliter of the final culture medium), sulfanilamide in a concentration of 1 per cent caused a continuous decrease in the number of viable hemolytic enterococci. In contrast, after an initial decline, the number of viable microörganisms showed a steady increase in the control

broth for several days. It may be mentioned that this marked increase in the number of living bacterial cells in the control broth occurred in spite of the fact that the fermentation of maltose caused a change in the pH to the acid side, whereas no such change occurred in the sulfanilamide broth. It may be noted from figure 1 that viable microörganisms could no longer be demonstrated in the sulfanilamide broth after two days' incuba-

BACTERIOSTATIC ACTION OF
SULFANILAMIDE (1%)
ON
HEMOLYTIC ENTEROCOCCUS
IN
6.5% NaCl-¼% MALTOSE BROTH
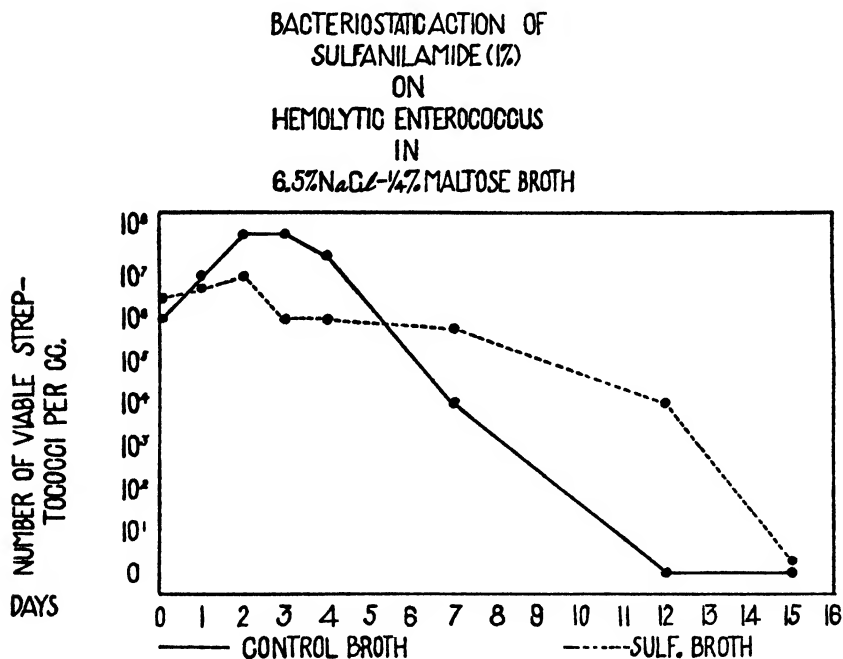
FIG. 2

tion, while in the control broth viable microörganisms were present even after an incubation period of 12 days. Similar results were obtained with somewhat larger inocula. In one particular experiment (10,000 viable streptococci per milliliter in the final culture medium), 1 per cent sulfanilamide caused a steady decrease in the number of viable streptococci; viable microörganisms could no longer be demonstrated after an incubation period of 4 days. In contrast, the number of streptococci

markedly increased in the control broth and viable micro-organisms were present even after more than 11 days' incubation.

The results of an experiment in which rather large inocula (1,000,000 to 5,000,000 microörganisms per milliliter of the final culture medium) were used, is presented in figure 2. It may be seen that 1 per cent sulfanilamide did not exert a marked bacterio-static action on the hemolytic enterococcus. It is interesting

BACTERIOSTATIC ACTION OF
SULFANILAMIDE (1%)
ON
HEMOLYTIC ENTEROCOCCUS
IN
6.5% NaCℓ-¼% MALTOSE BROTH
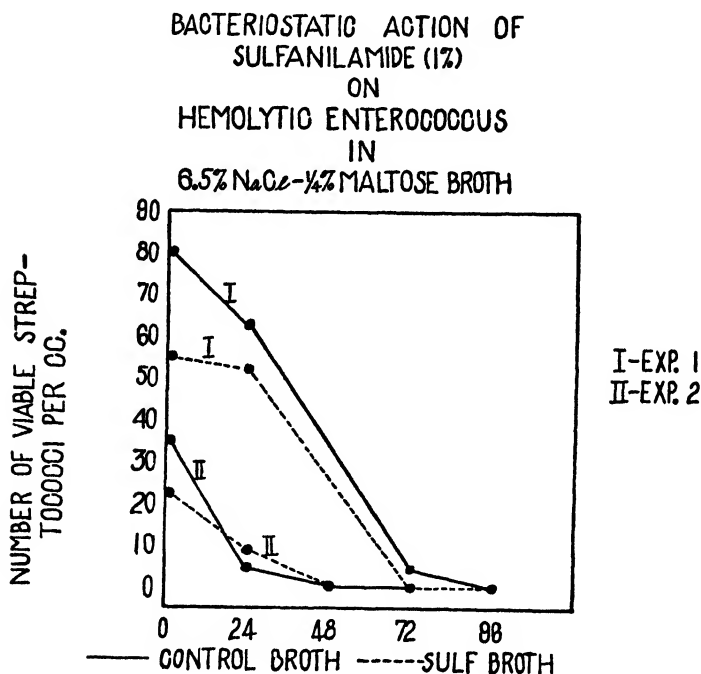
FIG. 3

to note that in this experiment viable streptococci could be demonstrated in the sulfanilamide broth for a few days longer than in the respective control broth.

An interesting observation was made when the action of sulfanilamide upon very small numbers of bacterial cells (20 to 80 viable microörganisms per milliliter of the final culture medium) was tested. It was found, as shown in figure 3, that 1 per cent sulfanilamide did not markedly enhance the rate

of decrease in the number of viable enterococci in the sodium chloride broth. It may be emphasized that this inoculum was too small to cause a noticeable growth even in the control broth. Essentially the same results were obtained with strains of non-hemolytic enterococci; namely, definite bacteriostatic activity of sulfanilamide in a concentration of 1 per cent against suitable numbers of microörganisms in this sodium-chloride broth medium. Similarly to the above recorded experiments with hemolytic enterococci, sulfanilamide lacked definite bacteriostatic activity toward very large number of non-hemolytic enterococci and

TABLE 2

Bacteriostatic action of sulfanilamide on hemolytic enterococcus in ¼ per cent maltose phenol red broth

HOURS OF INCUBATION AT 37°C.	INOCULUM (48 HOUR BROTH CULTURE)							
	A, 1:125,000 0.1 mL.				B, 1:3,125,000 0.1 mL.			
	Concentration of sulfanilamide							
	1:100	1:1000	1:10,000	0	1:100	1:1000	1:10,000	0
18	−	+++	+++	+++	−	+	++	+++
24	−	+++	+++	+++	−	++	+++	+++
48	++++	++++	++++	++++	−	++++	++++	++++
72	++++	++++	++++	++++	−	++++	++++	++++
4 days	++++	++++	++++	++++	−	++++	++++	++++
1 week	++++	++++	++++	++++	−	++++	++++	++++

−, no visible growth; + to ++++, various degrees of visible growth.

failed to enhance the death rate of very small inocula. In the above described experiments the bacteriostatic action of sulfanilamide toward both hemolytic and non-hemolytic enterococci was tested in a culture medium containing a relatively high concentration of sodium chloride. Further experiments indicated that a certain degree of bacteriostasis may also be observed when the sodium chloride content of this culture medium was reduced from 6.5 to 0.5 per cent and the maltose content from 1 to ¼ per cent. Bacteriostasis was obtained only when very small numbers of bacterial cells were used for inoculation of the broth media. A representative example of such an experiment, in which the

bacteriostatic action of sulfanilamide in concentrations ranging from 1 to 0.01 per cent toward a strain of hemolytic enterococcus was tested, is given in table 2. In this particular experiment, 0.1 milliliter of 1:125,000 and 1:3,125,000, dilution of a 48-hour broth culture, respectively, was used as the inoculum. It may be seen from this table that sulfanilamide in a concentration of 1 per cent only delayed the growth of the larger inoculum, but completely inhibited it for one week with the smaller inoculum.

BACTERIOSTATIC ACTION OF SULFANILAMIDE (1%) ON HEMOLYTIC ENTEROCOCCUS AT 37°C. AND 43°C. IN ¼% MALTOSE PHENOL RED BROTH
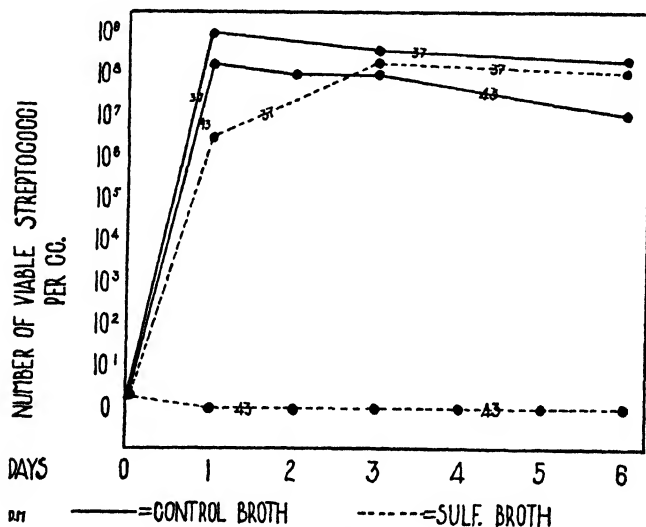
FIG. 4

Sulfanilamide in a concentration of 0.1 and 0.01 per cent had no noticeable growth inhibitory effect with the larger inoculum and caused only slight retardation and inhibition of growth with the smaller inoculum.

White and Parker (1938) and White (1939) reported a marked increase in bacteriostatic and bactericidal activity of sulfanilamide toward hemolytic streptococci *in vitro* when the temperature of incubation was raised from 37 to 39 or 40°C. A similar enhance-

ment of bacteriostatic activity of sulfanilamide toward members of the enterococcus group was observed when the temperature was increased from 37°C. to approximately 43°C.   In order to test quantitatively the effectiveness of sulfanilamide at different temperatures, the following experiment was carried out.   The culture medium employed was ¼ per cent maltose phenol-red broth with and without 1 per cent sulfanilamide.   A very small inoculum of hemolytic enterococcus was used.   Two parallel sets were incubated at 37 and 43°C., respectively.   The number of viable microörganisms was determined at various intervals (fig. 4).

It may be seen from figure 4 that sulfanilamide in a concentration of 1 per cent at 37°C. only delayed and slightly inhibited the growth of the hemolytic enterococcus.   In contrast, at 43°C. 1 per cent sulfanilamide completely prevented the growth of the microörganisms.   It is interesting to note that after 24 hours' incubation, viable bacterial cells were no longer demonstrable in the sulfanilamide broth, incubated at 43°C.   It is important to mention that the growth of the hemolytic enterococcus in the control broth at 43°C. was only slightly inhibited in comparison to that obtained at 37°C.   It may be concluded from this experiment that the marked enhancement of bacteriostatic activity can not be explained solely on the basis of a growth inhibitory effect of higher temperature.   These observations with members of the enterococcus group parallel those of White and Parker (1938) and White (1939) with hemolytic streptococci.

DISCUSSION

The above-reported experiments revealed that sulfanilamide in a concentration of 1 per cent may exert definite bacteriostatic activity *in vitro* toward members of the enterococcus group, including both hemolytic streptococci Group D and non-hemolytic enterococci.   In order to demonstrate complete inhibition of growth of the enterococci by sulfanilamide, it was found necessary to use relatively small numbers of microörganisms, high concentrations of sulfanilamide, special culture media or certain conditions of incubation such as higher temperature.   There

can be little doubt, however, that the growth of hemolytic strepto-
cocci of Group A is far more readily inhibited by sulfanilamide
in a concentration of 1 per cent than is the growth of enterococci.
In this connection, it is interesting to note that the members of
the enterococcus group, generally, are more resistant toward
various physical and chemical influences than are hemolytic
streptococci of other groups.   Thus, in contrast to hemolytic
streptococci of Groups A, B, and C, the members of the entero-
coccus group may survive when exposed to a temperature of
60°C. for 30 minutes.   Furthermore, enterococci may grow in
culture media containing 6.5 per cent sodium chloride, in broth
of a pH of 9.6, or containing 0.1 per cent methylene blue.   In
addition, the members of the enterococcus group may even grow
at temperatures of 10 and 45°C.   It is conceivable that this
general resistance of the enterococcus group may account, to a
certain extent at least, for its lesser susceptibility to the action
of sulfanilamide.

The members of the enterococcus group are characterized by
another property that is not shared by hemolytic streptococci
of Groups A, B, and C, namely, their strong reducing power.
The question arises whether or not this marked reducing activity
of enterococci may possibly interfere with the action of these
chemotherapeutic substances of the sulfanilamide group.

It was shown in the experiments reported above that sulfanil-
amide in a concentration of 1 per cent may not only inhibit the
growth of suitable numbers of enterococci, but also may cause a
more or less rapid decrease in the number of viable microör-
ganisms.   The question has to be considered whether this effect
is due to a true bactericidal action of the drug or whether it
is the result of bacteriostasis, representing the death rate of a
suspension of microörganisms that is not in the phase of multi-
plication.   The experiments in which the number of micro-
organisms used for inoculation was too small to cause visible
growth in the control broth, revealed that sulfanilamide in a
concentration of 1 per cent did not markedly enhance the rate
of decrease of viable bacterial cells.   This may indicate that
under the conditions of these experiments no true bactericidal
action took place.   However, it must be kept in mind that any

change in the experimental conditions may materially influence the results of chemotherapeutic experiments. In preliminary experiments the effect of sulfanilamide on enterococci suspended in saline solution was tested. This order of experiment was chosen in order to keep active multiplication of the microörganisms at a minimum. It was found that at 43°C. sulfanilamide in a concentration of 1 per cent has no striking effect on the rate of decrease in the number of viable bacterial cells.

SUMMARY

A quantitative study on the action of sulfanilamide *in vitro* upon members of the enterococcus group revealed the following results:

1. Sulfanilamide in a concentration of 1 per cent may be bacteriostatic toward suitable numbers of both hemolytic and nonhemolytic enterococci in a maltose broth medium containing 6.5 per cent sodium chloride.

2. Sulfanilamide may cause a rapid decrease in the number of viable enterococci, whereas a marked increase takes place in the control broth.

3. The bacteriostatic activity of 1 per cent sulfanilamide in the same culture medium is negligible or absent when very large number of bacterial cells are used for inoculation.

4. Sulfanilamide in a concentration of 1 per cent failed to enhance the rate of decrease in the number of viable microorganisms in this culture medium when the inoculum was too small to cause growth in the control broth.

5. A certain degree of bacteriostasis by sulfanilamide could also be demonstrated when the sodium chloride content of the culture medium was reduced from 6.5 to 0.5 per cent and the maltose content from 1 to ¼ per cent. In this culture medium sulfanilamide in a concentration of 1 per cent inhibited the growth of very small inocula of enterococci only. In concentrations from 0.1 to 0.01 per cent sulfanilamide only delayed the growth of hemolytic enterococci but did not completely prevent it.

6. At 43°C. sulfanilamide in a concentration of 1 per cent is markedly more bacteriostatic toward hemolytic enterococci than at 37°C. The sulfanilamide broth incubated at 43°C. may become rapidly sterile at a time when the control broth incubated

at 43°C. and sulfanilamide broth incubated at 37°C. contain large number of viable microorganisms.

7. It may be concluded from these *in vitro* experiments that sulfanilamide in a concentration of 1 per cent may be definitely bacteriostatic toward both hemolytic and non-hemolytic enterococci. Sulfanilamide may also enhance the rate of decrease in the number of viable enterococci *in vitro*.

The author wishes to express his appreciation to Mrs. M. L. Short and Mr. D. McConville for the help rendered in these experiments.

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YEASTS OCCURRING ON GRAPES AND IN GRAPE PRODUCTS IN CALIFORNIA

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The yeasts found on the grapes and in the wines of California have received little attention from the botanical point of view, although the viticultural industries of the area are comparatively old. Holm (1908) incompletely described several yeasts obtained from California grapes designating them by number or as *Saccharomyces ellipsoideus* (*Bioletti* I Nov. Var.). He concluded that yeasts found on grapes produced in regions remote from wine-making operations are inactive in respect to the formation of alcohol and that many are detrimental to wine by producing films, turbidity, and unpleasant flavors. According to Bioletti (1911) *S. ellipsoideus* is the most common wine yeast and *Saccharomyces apiculatus* the most common pseudo-yeast occurring on grapes. Bioletti and Cruess (1912) briefly described the following yeasts obtained from grapes produced in the Davis, Acampo and Contra Costa vineyard areas of California: *Saccharomyces apiculatus*, *Saccharomyces ellipsoideus*, "wild yeast (*Saccharomyces* sp.)," and other "wild yeasts" characterized by film formation and slow fermentation. Cruess (1918) described cultures of *S. ellipsoideus*, *Saccharomyces pastorianus*, *Willia anomalus*, *S. apiculatus*, *Mycoderma* and *Torula* obtained from California grapes but failed to obtain cultures of *Saccharomyces ludwigii*, *Saccharomyces cerevisiae*, *Saccharomyces malei*, or *Saccharomyces marxianus*. Grapes from the wine-producing area of Ripon, Tulare, Fresno and Contra Costa regions yielded more true yeasts than grapes obtained from the remote areas of Davis and El Centro, and ripe grapes contained greater numbers of yeasts than green grapes. Suminoe (1928, 1930) reported on wine yeast isolated from California wine mash.

EXPERIMENTAL PROCEDURE

Grapes (at various stages of maturity ranging from firm green to soft ripe, and in a few instances, the raisin stage), grape must, and pomace were collected in various grape and wine-producing areas primarily in Central California. In addition a few samples of cloudy bottled wine, vineyard soil and grape leaves were included. All wine samples were from unsulphited naturally fermented musts. All samples were collected in sterile jars and examined as quickly as possible. The grapes were crushed in the original containers and incubated at room temperature. Sterile 10° Balling grape juice was added if needed. Streaks on wort agar were made as soon as fermentation started, and in some instances after it had ceased. Purification was accomplished by three platings on agar with isolated colonies in each case picked to grape juice. Cultures were identified in accordance with the procedures and systems of Stelling-Dekker (1931) and Lodder (1934). Color comparisons were made with the charts of Ridgway (1921). Carrot, beet, potato and cucumber wedges and Gorodkowa agar slants were used as sporulation media. Other media were used only when spores failed to form on those listed. In a few instances sporulation occurred on cucumber wedges only. The utilization tests with anascosporogenous yeast were conducted with synthetic liquid medium rather than auxanogramic plates because the liquid method expedited the determinations and gave results comparable to those obtained by the auxanogramic method.

Considerable difficulty was experienced in the differentiation of the families *Rhodotorulaceae* and *Torulopsidaceae* because of the inconclusiveness of the carotene crystallizing experiments. Numerous attempts to obtain crystals from *Rhodotorula rubra* (Demme) Lodder were often doubtfully positive. Prolonged storage (6 weeks) of the cells in the Molisch solution seemed to give better results. It was necessary to rely to a considerable extent on visible color differentiation as well as on the carotene crystal method to differentiate the two families.

A large number of the cultures differed slightly from forms described by others. In view of the recent contributions of

Satava (1934), Winge (1934) and Winge and Laustsen (1937, 1938) which have established the occurrence of haploid and diploid generations as well as hybridization in yeast, it is inadvisable to establish new varieties or species unless the differences are considerable. A large number of organisms showing only slight variations from described species have been included in existing species or varieties. Such differences, however, are discussed in the text as a matter of record.

<div align="center">

RESULTS

Sporulating yeasts

</div>

Genus *Saccharomyces:* Ninety-five of 118 cultures of *Saccharomyces* were identified as *S. cerevisiae* Hansen and only 4 as *S. cerevisiae* var. *ellipsoideus* (Hansen) Dekker. Wine yeasts are normally included in the *ellipsoideus* variety of *S. cerevisiae* on the basis of cell shape and size, but it is very difficult at times to distinguish the variety on cell morphology alone. Since many of the cultures identified as *S. cerevisiae* Hansen were isolated as the predominating organism from naturally fermenting musts it is quite possible that they are enologically different from the type cultures of *S. cerevisiae* and *S. cerevisiae* var. *ellipsoideus*, although the physiological characteristics used in taxonomy are similar.

Three cultures (16B1, 37B1, 74A1) were termed *S. oviformis* Osterwalder, although they differ slightly in slant culture characteristics. *S. oviformis* is described by Stelling-Dekker as producing smooth, slightly glossy slant cultures with waxy borders. The slant culture of 74A1 is similar to that of *S. oviformis* but that of 16B1 differs by having a finely contoured surface and lacerate border. The streak of 37B1 has an entire border and a slightly vesicular surface. Physiological characteristics corresponded in detail to those of *S. oviformis* proper.

Fifteen cultures closely resembling *S. chodati* Steiner were isolated. *S. chodati* ferments sucrose slowly and has slightly wrinkled slant cultures, whereas the cultures isolated ferment sucrose rapidly and possess smooth slant cultures. Other characteristics were similar.

A single culture (119A1), which corresponded exactly to the description, was identified as *S. carlsbergensis* Hansen.

Subgenus *Zygosaccharomyces:* Cultures were assigned to *Zygosaccharomyces* only when conjugation preceded spore formation. In most cases sexuality was observed rather infrequently. In several instances conjugating cells formed only sterile "dumbbells" on beet wedges. These organisms were assigned to the subgenus *Saccharomyces* rather than the subgenus *Zygosaccharomyces* although Stelling-Dekker assigned *Z. japonicus*, which displayed similar abortive sexual characteristics, to the subgenus *Zygosaccharomyces*. This culture, however, had been maintained in the laboratory for a long period of time.

Two cultures (31A3 and 40A1) were identical with *Z. priorianus* Klöcker and 11 were similar. Eight other cultures were included in this species although the vegetative cells were slightly smaller and the slant cultures somewhat more smooth than those of the described culture. Our cultures utilized alcohol slowly, whereas *Z. priorianus* does not.

Cultures 48C2 and 213 have been termed *Z. barkeri* Saccardo and Sydow although they ferment sucrose and raffinose more rapidly than the organisms described by Stelling-Dekker.

Genus *Pichia:* Four species of *Pichia* were represented as follows: *P. alcoholophila* Klöcker (83B1), *P. membranaefaciens* Hansen (83A1F), *P. neerlandica* Lodder, and *P. belgica* (Lindner) Dekker (220). Although culture 83B1 was designated as *P. alcoholophila*, it varied from the description of this organism by producing a finely wrinkled slant culture and failing to produce definitely allantoid cells. The two cultures of *P. neerlandica* differed from the organisms described by Lodder in failing to produce slant cultures with serrate edges.

Subgenus *Zygopichia:* Two cultures (represented by 53B3TW) very similar to *Z. chevalieri* (Guilliermond) Klöcker were isolated from wines. These cultures liberate their ascospores soon after formation so that the sexual character is easily overlooked. A few spores with very small brims have been noticed, but the majority of the spores are hemispherical.

Genus *Debaryomyces:* Relatively few cultures of *Debaryomyces*

were obtained from grapes and grape products although species of this genus have been found commonly in California on other products by Mrak and Bonar (1939). Two cultures (7A1 and 7B1) similar to *D. globosus* Klöcker differed by failing to produce a pellicle on alcohol medium. A single culture (50B1) was similar to *D. maturuchoti* Grigoraki and Peju except for slightly smaller cells and less moist slant culture.

Genus *Hansenula*: Although species of *Hansenula* have been observed on various food products produced in California, only four were isolated from grape products. One (192) is very similar to *H. anomala* var. *sphaerica* (Nageli) Dekker except that the streak culture is slightly punctate rather than mealy.

Culture (216-H) is similar to *Hansenula panis* Castelli except that the spores are globose rather than hat-shaped, and alcohol and nitrate are not utilized. These differences are sufficient to warrant specific segregation which, however, will not be made here since a study of this entire genus is being made by C. L. Bedford of this Laboratory. Designation of species and varieties of this genus are likewise withheld for the same reason.

Genus *Torulaspora*: A single culture (56B4) of *Torulaspora rosei* Guilliermond was isolated from grapes. This culture is identical with that described by Stelling-Dekker, but is unusual in forming frequently three spores per ascus and numerous rather long copulation trial tubes.

Genus *Hanseniaspora*: This genus is commonly found on grapes and musts in Europe. Bioletti and Cruess (1912) discussed several cultures of *Hanseniaspora* obtained from California grapes and musts but failed to indicate the specific designation. Eleven cultures were found to be almost identical with *H. guilliermondii* Pijper. In a few instances slight variations (believed to be insignificant) were observed in alcohol and nitrate utilization.

Genus *Kloeckeraspora*: The validity of this genus is questioned; Niehaus (1932), Castelli (1935a) and Dvornik (1938). It is not our purpose to discuss this question or to interpret the morphological significance of the central bodies termed spores by Niehaus and Dvornik and vacuoles by Castelli. The culture

(95B1) studied was morphologically and physiologically identical with *Kloeckeraspora uvorum* Niehaus. The spherical bodies, illustrated by Niehaus, were observed in approximately five per cent of the cells from beet and potato wedge cultures stored at room temperature for three weeks. In a few instances rough surfaces were observed on these bodies.

Non-sporulating yeasts

Less than half of the cultures studied failed to produce, in 60 days, ascospores on various media. The Stantial (1935) procedure was tried and gave negative results when ascospores were not formed on the common sporulation media. *Torulopsis, Mycoderma, Kloeckera* or *Candida* were most frequent although species of *Rhodotorula, Asporomyces* and *Schizoblastosporion* were found. Difficulty was experienced in identifying the non-sporulating yeasts because of slight variation in physiological and morphological characters.

Genus *Torulopsis:* Culture (98A2-2) is similar to *Torulopsis lipofera* (den Dooren de Jong) Lodder in all characteristics except that the utilization of urea is somewhat doubtful and the cells are not quite as long as those of the organism described by Lodder.

Three cultures (41B3R, 58B1, 116A) were similar to *T. pulcherrima* (Lindner) Saccardo but formed slant cultures that were smooth and dull rather than shiny. A second group of six cultures, typified by 31A1W, failed to show definite utilization of alcohol and urea. A third group of 10 cultures, typified by 124A, differed from *Torulopsis pulcherrima* in forming dull smooth slant cultures.

Three cultures of non-film-forming budding yeast with globose to spherical cells (represented by 80C2) were assigned to the genus *Torulopsis* although they were strongly fermentative and in this way distinctly different from any of the described species. These organisms are described as *Torulopsis californicus* Nov. Sp. A fourth culture (50C1) is also strongly fermentative but differs from the aforementioned cultures in alcohol and nitrogen utilization characteristics and the partial fermentation (as determined in the van Itterson-Kluyver apparatus) of raffinose. This culture is described as *Torulopsis fermentans* Nov. Sp.

Torulopsis californicus Nov. Sp.

Cells mostly globose to spherical. Cell size in 24-hour wort culture range (3.5–5.25 μ) x (4.5–6 μ) and average 3.5 x 5.25 μ. From three-day wort culture range (5.25–7 μ) x (5.25–8.75 μ) and average 6 x 6.5 μ. Ferments glucose, levulose, mannose, galactose, maltose, and sucrose, not raffinose or lactose. Asparagin, ammonium sulphate, urea and peptone utilized slowly, nitrate not. Good growth when alcohol is the only source of carbon. Slant cultures cartridge buff, smooth to slightly punctate, dull, dry, and pulvinate, with entire to undulate borders. Giant colony cartridge buff, smooth, dull dry, and convex with entire borders. Gelatin not liquefied in 70 days.

Torulopsis fermentans Nov. Sp.

Cells globose to spherical. Cell size in 24-hour wort range (3.5–5.75 μ) x (4.4–8.75 μ) and average 4.5 x 5.75 μ. From three-day wort culture range (3.5 7 μ) x (4.5 8.75 μ) and average 6 x 7 μ. Ferments glucose, levulose, mannose, galactose, maltose, sucrose and ($\frac{1}{4}$ only) raffinose. Lactose not fermented. Peptone and alcohol are utilized. Asparagin, urea, ammonium sulphate, and nitrate not utilized. Slant culture pale pinkish cinnamon, smooth, dry, dull, and raised with entire borders. Giant colonies pale pinkish buff, smooth, dull, dry, flat crateriform cone with entire borders. Gelatin not liquefied in 70 days.

Genus *Mycoderma:* Few organisms corresponding to the genus as defined by Leberle (1909) and Lodder (1934) were found. These were similar to *Mycoderma cerevisiae* Desm. Several cultures identified as *Candida* were similar to *Mycoderma* in all respects except for the formation of pseudomycelia on Rivalier and Seydel slides.

Genus *Kloeckera:* The majority of the 16 cultures, typified by 104A1, belonging to this genus are very similar to *Kloeckera lindneri* (Klöcker) Janke. Slight variations were observed in cell size and streak culture characteristics. In a few cases the cell size range varied slightly from those given by Lodder. The streak cultures ranged in color from avelleneous to wood brown and were dry but glistening. Culture 120A1 was comparable to *Kloeckera africana* (Klöcker) Janke in cell size and most other characteristics although it liquefied gelatin slightly in 60 days.

Genus *Asporomyces:* According to Chaborski (1919) this genus

is characterized by forming abortive conjugation tubes on Gorodkowa agar without producing ascospores. *Asporomyces asporous* Chaborski was isolated from bananas and was characterized by fermenting glucose, levulose, mannose, sucrose, maltose and raffinose. Unfortunately Chaborski's culture was lost and hence is not available for comparison with the cultures isolated from grapes. Cultures 96B3W and 96B3R produce abortive conjugation tubes in 10-per-cent sucrose solution and on beet and cucumber sporulation wedges but not on Gorodkowa

FIG. 1. VEGETATIVE CELLS OF ASPOROMYCES UVAE NOV. SP. SHOWING ABORTIVE CONJUGATION TUBES

agar slants. If the genus *Asporomyces* is acceptable, the substrate in or on which these abortive tubes form should not be limited to the single sporulation medium but should include all sporulation media used in the identification of yeast. For this reason the cultures (96B3W and 96B3R) isolated are included in this genus and described as *Asporomyces uvae* Nov. Sp. Cells showing the abortive conjugation tubes are shown in Figure 1.

Asporomyces uvae Nov. Sp.

Cells in young wort culture globose, ellipsoidal, spherical or pyriform. Cell size in 1 and 3-day wort culture, range (2.5 5.25 μ) x (3.5 10.5 μ)

and average 4.5 x 6 μ.   Thin pellicle and ring appears on wort culture
after 4 days.   Cells form abortive conjugation tubes on cucumber and
beet wedges.   Glucose, fructose, and mannose fermented slowly.
Sucrose, maltose, lactose and raffinose not fermented.   Utilization of
alcohol in synthetic medium doubtful.   Peptone, asparagin, ammonium
sulfate and urea assimilated, nitrate not.   Slant cultures ivory yellow
(sometimes with a pinkish tint), smooth, dull, dry, convex with lobate-
lobulate border.   Giant colonies cartridge buff, smooth, moist and
glistening in the center, dull and dry at the border, flat with entire
border.   Gelatin liquefied in 60 days.

Genus *Schizoblastosporion:*[1] A single culture was isolated from
grape leaves.   Bud-fission was quite evident in actively growing
wort cultures when the organism was first isolated.   After pro-
longed cultivation (3 years) on wort agar, however, this char-
acteristic was much more difficult to observe.   Observations
made on an authentic culture of this type species revealed that
the bud fission character was likewise not very evident in this
culture which had been held in culture for a number of years.

The culture isolated differed very slightly from *Schizoblasto-
sporion starkeyi-henricii* Ciferri (98A1C) in slant and giant colony
characteristics and cell size.   The streak culture of our culture
was relatively dry and dull.   The giant colony was not raised
in the center, and the cells were slightly larger than those of a
type culture.

Mycotoruloideae: The taxonomy of the organisms belonging
to this subfamily is so confused at present that it is useless to
attempt to identify species.   Langeron and Guerra (1938)
grouped the genera of the subfamily *Mycotoruloideae* into the
single genus *Candida*, including the various species studied in
seven groups.   Our cultures fit into group five, characterized
by fermenting glucose and levulose only, and group seven, char-
acterized by failing to ferment any sugars.   Recently Diddens
and Lodder (1939) proposed a scheme for classifying the or-
ganisms of the subfamily *Mycotoruloideae* whereby the subfamily
is comprised of the genera *Candida* Berkh. and *Trichosporon*
Behrend.   They divide the genus *Candida* into four groups.

[1] Langeron and Guerra (1938) have recently reduced this genus to synonomy
with *Candida.*

Several of our organisms fit into the *Candida albicans* group (group 1) characterized by pseudomycelia, varied forms of blastospores, no pellicle formation on wort, creamy or membranaceous appearance of the cultures which are never flat or dull. Most of the cultures, however, fit into the *Candida krusei* group (group 2) because of the presence of pseudomycelia, characteristic blastospores and pellicle formation, and the fermention of glucose, fructose and mannose, although fermentation has not been ob-

TABLE 1

Occurrence of yeast genera in grapes and grape products in California

	FRESH GRAPES	MUSTS AND NEW WINES	CLOUDY BOTTLED WINE	POMACE LEAVES OR SOIL	TOTAL NUMBER OF CULTURES
Saccharomyces	43	58	14	3	118
Zygosaccharomyces	7	6			13
Hanseniaspora..................	9	2			11
Kloeckeraspora.......	1				1
Pichia.........................	2	1	1	2	6
Debaryomyces	3				3
Hansenula.....................	2	1	1		4
Zygopichia................... ..		1	1		2
Torulaspora	1				1
Torulopsis.....................	17	4	2	2	25
Mycoderma.	2	3		1	6
Kloeckera...........	15	1			16
Rhodotorula......	6				6
Asporomyces...................	2				2
Schizoblastosporion............				1	1
Candida.......................	18	3	2	3	26

served in a few cultures. Only one culture possessed the characteristics of *Brettanomyces* (group 4) characterized by varied-shaped blastospores; a creamy appearance which is never dull but develops a brown tinge, and the fermentation of several sugars.

Grouping the numerous ill defined genera of the subfamily *Mycotoruloideae* into one or two well defined genera by the above authors is a desirable step forward in the taxonomy of this group, and their suggested changes should be adopted to facilitate the establishment of a reliable system for specific taxonomy. Specific

designation of our cultures is withheld pending publication of a monograph on the subfamily *Mycotoruloideae* by Diddens and Lodder.

Genus *Rhodotorula:* Six cultures of red-colored non-sporulating yeast were isolated. It cannot be stated definitely, however, that any of these organisms belong to the genus *Rhodotorula* because of the difficulty experienced in obtaining reproducible results with the Molisch technique for the crystallization of carotene, as previously mentioned. Further consideration is being given this particular problem. In view of these difficulties the cultures designated as *Rhodotorula* have been judged so on the basis of morphological and physiological characteristics and by comparison with an authentic culture.

Three of the organisms, similar to 56B2S, are similar to *R. sanniei* (Ciferri and Redaelli) Lodder except that the cells of our cultures are slightly smaller and the slant culture is smoother than those of *R. sanniei.* The other 3 cultures, represented by 114A3, are similar to *R. glutinis* (Fres) Harrison as described by Lodder.

DISCUSSION

The 241 cultures of yeasts fell into the genera presented in table 1. The genus *Saccharomyces* was obtained most frequently, and the next most common group consisted of the apiculate yeasts, *Kloeckera*, *Kloeckeraspora*, and *Hanseniaspora*. The latter genera, however, were obtained more commonly from grapes than from musts or wines. Cultures of *Torulopsis* and *Candida* were obtained commonly from grapes. This distribution is in general agreement with the findings of De Rossi (1935), Castelli (1935b, 1938), and Verona and Luchetti (1936) in the wine-producing regions of Europe. The organism most commonly obtained from cloudy bottled wine was *Saccharomyces cerevisiae* although occasional cultures of *Candida*, *Pichia*, *Zygopichia*, and *Torulopsis* were isolated. No definite correlation between the distribution of any genera or species with a particular district has been observed.

Cultures of the organisms, especially described in this report,

have been retained by the authors and have been deposited in the Centraalbureau voor Schimmelcultures and the American Type Culture Collection.

SUMMARY

Two hundred and forty-one cultures of yeasts were isolated from California grapes and grape products. The 159 ascospore forming yeasts included: *Saccharomyces cerevisiae* Hansen, *S. cerevisiae* var. *ellipsoideus* (Hansen) Dekker, *S. oviformis* Osterwalder, *S. chodati* Steiner, *S. carlsbergensis* (Hansen); *Zygosaccharomyces priorianus* Klöcker, *Z. barkeri* Saccardo; *Pichia alcoholophila* Klöcker, *P. membranaefaciens* Hansen, *P. neerlandica* Lodder, *P. belgica* (Lindner) Dekker; *Zygopichia chevalieri* (Guilliermond) Klöcker; *Debaryomyces globosus* Klöcker, *D. matruchoti* Grigoraki and Peju; *Hansenula anomala* var. *sphaerica* (Nageli) Dekker; *Torulaspora rosei* Guilliermond; *Hanseniaspora guilliermondii* Pijper and *Kloeckeraspora uvorum* Niehaus. The 82 imperfect yeasts included: *Torulopsis lipofera* (den Dooren de Jong) Lodder, *T. pulcherrima* (Lindner) Saccardo; *Mycoderma cerevisiae* Desmazieres; *Kloeckera lindneri* (Klöcker) Janke, *K. africana* (Klöcker) Janke; *Schizoblastosporion starkeyi-henricii* Ciferri; *Rhodotorula sanniei* (Cif. and Red.) Lodder, *R. glutinis* (Fres) Harrison and several species of *Candida*.

Two new species of *Torulopsis* were described as: *Torulopsis californicus* Nov. Sp. and *Torulopsis fermentans* Nov. Sp. Two new cultures of the lost genus *Asporomyces* were isolated and described as *Asporomyces uvae* Nov. Sp.

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THE USE OF SILICA GELS FOR THE CULTIVATION OF HALOPHILIC ORGANISMS

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The cultivation of that group of organisms known as the halophiles has claimed the attention of industrial bacteriologists for some time. Various types of media have been proposed but none have proved completely satisfactory. Rice starch paste, hide and fish digest made solid with agar, coagulated eggs and the classic nutrient agar containing 3 molar sodium chloride have been proposed and used by many workers with indifferent results. The most serious criticisms of these media are (1) slow, scanty growth; (2) colonies small and hard to see; (3) high pouring temperatures of the agar mixtures; (4) peptization of the agar; (5) difficulty in clarifying, and (6) irregularities in the results.

Silicic acid jellies have been used since 1890 for the cultivation of soil organisms. Compared with the media mentioned previously, these gels offer larger pore size, lowered pouring temperatures and the possible presence of more free water to carry nutrients to the cells. With these characteristics, silicic acid gels may provide a particularly advantageous environment for the cultivation of salt-tolerant organisms; and it was to test this possibility that the following experiments were undertaken.

Various combinations of inorganic salts and organic materials were prepared in testing the usefulness of the medium. The nutrient salts used by Hanks and Weintraub (1936) were incorporated in the gels together with such organic materials as cystine, peptone, gelatin, and glucose. The sodium chloride content was brought as near as possible to 3 molar, which is accepted as necessary for the growth of true halophilic organisms.

A trace of bismuth ion was added, since from previous experiments with agar it seemed that there might be either a stimulation of growth of organisms, or production of colonies more easily visible due to the precipitation of black BiS by the decomposition of the sulphur compounds in the medium, or both.

The final combination of materials giving the highest salt content, greatest stability, greatest clarity, and capable of supporting growth, together with the conditions necessary in the preparation of the gels are as follows:

<center>*Solution A*</center>

Ammonium Sulfate	1 gram
Potassium dihydrogen phosphate..	0.2 gram
Dipotassium hydrogen phosphate...	0.8 gram
Glucose	20 grams
Cystine....	1 gram
Peptone	5 grams
Iron sulfate	.0001 gram
Bismuth citrate*	0.15 gram
Hydrochloric acid conc.	20 ml.
Saturated Sodium Chloride Solution	500 ml.

> ᵇ Hunter (1938)

<center>*Solution B*</center>

95 grams Sodium Silicate, Bakers 40° Bé, in 500 ml. 1.5 per cent sodium chloride solution

Baker's sodium silicate solution is specified since it was found that there are various grades and concentrations of this product on the market which did not produce clear jellies. The silicate must be clear and without a precipitate. Preparations from other companies are being studied, but not enough work has been completed to warrant an opinion as to their possible use. The silicate solution should be stored in a wax-lined flask and is preferably made up fresh for each batch of gel.

The quantities of silicate and acid are so calculated that equal portions of these solutions produce a suitable gel which sets rather slowly, is clear, and contains 14.5 per cent salt. Higher concentrations of silicate produce gels which set too rapidly, with formation of a precipitate. More salt cannot be added to the

silicate solution due to the formation of insoluble silicic acid which renders the medium unsuitable for quantitative work. A small amount of salt precipitates from the acid solution but is readily redissolved during the titration and contributes to the final salt concentration of the medium. The highest salt concentration which could be obtained was 14.5 per cent in the finished jelly.

The formation of the gel is carried out as follows: Ten ml. of the acid solution are pipetted into a small beaker, a few drops of bromthymol-blue indicator added, and the silicate run in slowly from a burette, with rapid shaking of the mixture until the color of the indicator just begins to change. This change occurs at approximately pH 7.2 and must not be overstepped, otherwise the gel will set too quickly and a flocculent precipitate will form in the medium. The mixture is then poured very quickly into a Petri dish containing the sample, mixed thoroughly and allowed to gel. Hanks and Weintraub have shown that these silica gels grow progressively more alkaline on standing, so that if they are poured at a pH of 7.0 or 7.2 they finally change toward pH 8.0. which is optimum for the growth of halophiles as shown in the results of many workers. There has been no attempt to sterilize these gels since it was felt that the strong acid and alkaline solutions would already be sterile, and chance organisms from the air would not grow in the high salt concentrations. Control plates made with each pouring of the gels have never shown any growth. Sterile petri dishes were used throughout, however. If the solutions are kept cold (4°C.), the gels set much more slowly and will permit better mixing with samples previously placed in the petri dishes.

If streak plates are to be made, it is best to prepare the gels 24 hours previously, incubate with porous clay tops, and then replace them with glass ones just before inoculation. This procedure dries off the surface water of syneresis and if care is exercised good streaks on the medium may be obtained.

Using the medium previously described, streak inoculations from various sources have been made. Agar plates containing the same nutrients and salt concentrations were inoculated

simultaneously and the time required for growth to appear was recorded. Table 1 gives the results of these investigations. It will be noted that in every case growth appeared on the silica plates some time before that on the agar plates. This growth was more profuse and of a more mucoid character, and decomposition of the glucose occurred in some cases, as evidenced by the change in color of the indicator to the acid side.

TABLE 1

Time required for visible growth of halophilic organisms on silica and agar gels

SAMPLE	TIME IN DAYS	
	Silica gel	Agar gel
Contaminated packing house salt:		
Number 1	4	11
Number 2.	4	7
Number 3	4	10
Number 4	7	>20
Crude solar evaporated sea salt from:		
Spain	4	>20
West Indies...	7	>20
Argentine..	3	10
South Africa...........	4	>20
California	7	>20
Brines from:		
Tainted steer hides	3	7
Tainted sealskin	4	15
Pure cultures of halophilic organisms:		
Number 1.......	4	15
Number 2	3	7

Attempts to use this medium for quantitative determination of organisms in brines from various sources have been made, but sufficient data have not been accumulated to make comparisons at this time. Sufficient work has been done, however, to note that colonies are formed and that their numbers correspond with the expected trends.

CONCLUSIONS

A new type of medium is proposed for the cultivation of halophilic organisms. This consists of a silicic acid jelly containing inorganic salts, cystine and peptone with traces of Fe and Bi ions. The medium is prepared by titrating, in the cold, sodium silicate and HCl solutions containing sodium chloride and the nutrient materials, and then adding the mixture to the sample. Concentrations of NaCl greater than 14.5 per cent have not proved practicable up to the present, but it is hoped that further study may develop them. Growth on this medium is more profuse, more rapid and more easily visible than on agar gels of the same salt and nutrient concentrations. The gels are stable under the conditions outlined, and may be streaked if proper care is exercised.

The use of this medium is suggested for all types of salted products, including skins and hides, fish, meats and vegetables, where studies of the flora of the materials or their brines are undertaken. It offers the distinct advantage of saving time and thus enables the worker to follow the rise and fall of bacterial populations while the brines are still in use.

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STUDIES ON CAPSULE FORMATION

III. Inhibition of Capsule Formation of Klebsiella pneumoniae (Friedländer's Bacterium) by an Agent Produced by a Soil Bacillus

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INTRODUCTION

The conditions under which *Klebsiella pneumoniae* forms its largest capsules were reported in an earlier publication (Hoogerheide, 1939). The underlying principle of that study was the hypothesis that capsular polysaccharide synthesis is brought about by the action of an enzyme system in the cell, the activity of which can be studied in the same way as that of other metabolic enzyme systems. In a second paper (Hoogerheide, 1940) the influence of electrolytes on this enzyme system was studied, and it was found that electrolytes, when added to the culture medium, bring about a marked inhibition in the production of capsular polysaccharide.

While engaged in attempts to isolate from bacterial sources possible enzymes capable of hydrolyzing the capsular substance of Friedländer's bacterium (similar to the enzymes described by Dubos and Avery (1931) and by Sickles and Shaw (1934, 1935), which digest the polysaccharides of *Pneumococcus* types I, II, III and VIII), we isolated several strains of a bacillus which appeared to have the remarkable property of excreting a soluble agent. By means of this agent, the bacillus is able to prevent Friedländer's bacterium from forming capsules. The present paper describes the isolation of this soil bacillus and the preparation of extracts containing the active principle.

Isolation of a sporulating bacillus from soil, capable of preventing capsule formation of Friedländer's bacterium

Small Erlenmeyer flasks containing a solution of 0.1 per cent K_2HPO_4, 0.1 per cent NH_4Cl, 0.05 per cent Mg_2SO_4, and 0.01 per cent (type B) Friedländer polysaccharide in tap water, were inoculated with small amounts of soil samples from different locations around Philadelphia and incubated at 37°C. After 1 or 2 days' incubation, growth could be observed. Several weekly transplants in sterile media of the same composition were made, after which the cultures were plated on peptone agar. Bacteria which had formed colonies of various appearances on these plates were isolated in pure culture and tested for their ability to grow in the polysaccharide medium. By this method, 24 strains of bacteria, belonging to several genera, were isolated from soil samples. These bacteria were able to multiply in a synthetic medium in which Friedländer polysaccharide, type B, was the only source of carbon. Although breakdown of this polysaccharide must have occurred, it was found that none of these strains would elaborate an enzyme which readily hydrolyzed the polysaccharide. Even after prolonged incubation (3 weeks), it was still possible by serological technique to demonstrate apparently unaltered polysaccharide.

With little hope of success, it was decided to determine whether or not the soil bacteria digest the natural capsules of Friedländer's bacterium. All of the 24 strains of soil bacteria were therefore inoculated into 4 per cent neopeptone-1 per cent glucose broth and incubated overnight. When growth had proceeded sufficiently the cultures were inoculated with Friedländer's bacterium. After 24 hours the presence or absence of capsules around Friedländer's bacterium was investigated microscopically using Burri's India ink method.

Of the 24 strains tested, there were three (which later proved to be identical) that appeared to inhibit encapsulation of Friedländer's bacteria very strongly, since practically no capsules could be detected; the remaining 21 strains did not inhibit the

capsule formation of Friedländer's bacterium. Since there was no indication that the growth of the Friedländer's bacterium had been retarded, to any extent, by the presence of the three inhibiting strains, it was decided to study more extensively this phenomenon of inhibition of encapsulation.
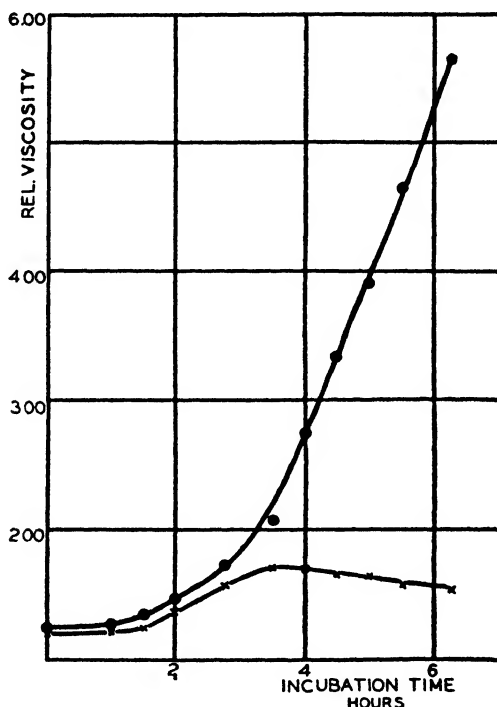
Fig. 1. Increase in Relative Viscosity of a Culture of Friedländer's Bacterium during 6 Hours' Incubation at 37°C. (Initial pH 7.5).
o——o in 4 per cent neopeptone-1 per cent glucose.
x——x in the same medium, but after previous 24-hour growth of 11M3.

The technique used to study the effect on average capsule size was the same as that described in the earlier papers of this series, namely, measuring the increase in relative viscosity during the growth of Friedländer's bacterium.

Figure 1 gives the results of an experiment in which the relative viscosity was measured during growth of Friedländer's bacterium in 4 per cent neopeptone-1 per cent glucose broth as

well as during growth in the same medium, inoculated 24 hours previously with a strain of the soil bacillus (11M3).[1] As may be seen from this figure, the curve representing the relative viscosity when soil bacilli are present is quite different from that representing the control culture. Three hours after inoculation, at which time the great increase in viscosity begins to manifest itself in the control culture, no similar increase was observed in the culture in which 11M3 had grown. A considerable decrease in viscosity occurred, notwithstanding vigorous growth of and fermentation by the Friedländer's bacterium. At the close of the experiment practically no capsules could be observed.

Similar results were obtained whether the growth of 11M3 had proceeded for 24 hours or for one month. The medium, after being readjusted to the proper pH, apparently contained a factor very unfavorable for capsule formation by Friedländer's bacterium.

The results obtained could not be attributed to an inhibition of growth due to exhaustion of the medium during the previous growth of 11M3 since such a culture, when filtered through a Seitz or Berkefeld filter, behaved as did the control. Apparently, filtration through Seitz EK pads or Berkefeld N candles removed the principle responsible for the inhibition of encapsulation.

When a culture of 11M3 in 4 per cent neopeptone-2 per cent glucose was centrifuged at high speed, a supernatant fluid was obtained which was practically free of bacteria (no growth was obtained when with a bent platinum needle a drop was streaked on peptone agar plates). Even when this supernatant fluid was incubated in a viscosity tube for 6 hours, under the same conditions observed in all of these experiments, no growth of 11M3 was observed. Nevertheless, such a culture medium, practically free of living cells, was very active in preventing capsule formation. Furthermore, it could be pasteurized, or even boiled, without loss of activity. Adsorption of this medium with Norit (Pfanstiehl), however, readily removed the active principle. We are probably dealing therefore with an excretion product of a soil bacillus which agent is heat-stable and readily adsorbable on

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[1] 11M3 is the laboratory designation of one of the three strains of isolated soil bacteria which inhibit encapsulation.

Norit and other adsorbents. By saturating the cell-free super-natant liquid of a culture of 11M3 in peptone-water with $(NH_4)_2SO_4$ a scum was formed, containing practically all of the active principle. By dialysis of this precipitate a purified, concentrated solution could be obtained.

The active principle is present not only in the supernatant fluid of the culture medium, but can be isolated from the washed precipitate also. When a suspension of 11M3 in distilled water was subjected to prolonged sonic vibration in a super-sonic oscillator, followed by high speed centrifugation, a cell-free solution of colloidal appearance was obtained. This solution contained the active principle which, when added to culture medium, would prevent capsule formation by Friedländer's bacterium. This colloidal solution appeared to be very stable and could be stored for months in the refrigerator without any loss of activity. However, when adjusted to pH 4.0 to 5.0 with acetic acid a precipitate was formed, containing all of the active principle. The precipitate redissolved when the pH was shifted either toward the alkaline side (pH 6.0) or to the acid side (pH 3.0). Such a precipitation can be repeated *ad libitum* without loss of activity. The active principle does not pass a cellulose dialyzing membrane; upon dialysis, however, a precipitate is formed, containing most of the active principle. Besides precipitation at pH 5.0, the active principle can be precipitated from colloidal solution by adding electrolytes. It can also be removed by treatment with Norit or colloidal iron (Merck).

The active principle is elaborated by the soil bacilli not only in the medium described but also during growth on any kind of medium, e.g., a 1 per cent gelatin solution containing the necessary inorganic salts. This indicates that the isolation on a medium containing Friedländer polysaccharide as the only source of carbon may have been just a coincidence. The three strains have been transplanted now for more than two years on the usual laboratory culture media without any sign of loss in activity. Furthermore, the active principle isolated from these soil bacteria not only prevents encapsulation of Friedländer's bacterium, type B, but also of strains of Friedländer's bacterium, type A.

The inhibitory principle is not an enzyme which destroys

capsular polysaccharide. When solutions of the active principle and of capsular polysaccharide are mixed, no breakdown occurs even after a prolonged incubation time. There is also no decrease in viscosity when the active principle is mixed with a heat-killed suspension of well-encapsulated Friedländer's bacterium. The active principle, therefore, presumably acts directly on the enzyme system responsible for the synthesis of capsular polysaccharide by inhibiting or even inactivating the enzyme. The amount of active principle necessary to inhibit encapsulation is very small: 0.005 mgm. of purified material per milliliter of culture medium is sufficient to cause an appreciable inhibition; 0.02 mgm. will almost completely prevent an increase in viscosity during growth. As was shown by making bacterial counts, the growth of Friedländer's bacterium is not affected, even when using 10 mgm. of active principle per milliliter of culture medium. Work is now in progress toward further purification and identification of the active material and will be published shortly.

Description of the organisms isolated

Colonies by growth on yeast-extract agar plates. Colonies are circular, 1 to 2 mm. diameter after 24 hours' incubation at 37°C., flat, smooth, entire edges, white, pearl-like glistening, sometimes becoming slightly yellowish-brown on further incubation. Mesophylic, strictly aerobic.

Morphology. Actively motile, gram-negative rods, single or in pairs, central spores, rods swollen during sporulation, endospores: ellipsoids.

Growth in nutrient broth. Moderate clouding, slight pellicle as well as sediment.

Carbohydrates. No fermentation.

Litmus milk. After 2 days: reduction of litmus, milk clotted, gradual peptonization, alkalinization.

Nitrate reduction. Active reduction and nitrite formation.

Gelatin liquefaction. Complete liquefaction of peptone-gelatin with considerable NH_3 formation.

Starch. No appreciable hydrolysis of starch.

Indole. No indole formation in tryptophane broth.

H_2S. After 1 week: growth on Pb acetate agar slightly brownish.

Catalase. Positive.

Isolation of the organism from soil

A soil suspension is made from about 1 gram soil in 10 ml. of water. This suspension is pasteurized for 10 minutes at 80°C. After sedimentation of the heavier soil particles 1 ml. of the supernatant fluid is used for inoculation, in a small Erlenmeyer flask, of 10 ml. of a sterile solution of 0.1 per cent K_2HPO_4, 0.1 per cent $(NH_4)_2SO_4$ and 0.1 per cent Friedländer polysaccharide, type B, in tap water.

After incubation for 3 days at 37°C. one loopful is transplanted to the same medium and this procedure repeated several times. After 3 or more transplants, plates are made on 0.1 per cent yeast-extract agar and the colonies, corresponding to the description above, isolated and tested for their ability to inhibit encapsulation of Friedländer's bacterium.

Because of the extreme complexity of the family Bacillaceae no extended attempt has been made to identify the isolated organism.

SUMMARY

Several strains of a gram-positive, spore-forming, aerobic bacillus have been isolated from soil. These organisms when growing on liquid media release a soluble agent capable of preventing Friedländer's bacterium, types A and B, from forming capsules.

A cell-free solution of the active principle was prepared from the supernatant fluid of a broth culture of this soil bacillus by adding $(NH_4)_2SO_4$, redissolving the scum and then dialyzing the solution. A similar solution was prepared from the centrifuged and washed bacteria by the sonic vibration of a bacterial suspension.

The active principle is heat-stable and is readily absorbed on Norit or colloidal iron. It has its minimum solubility at pH 5.0 and does not pass a dialyzing membrane.

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THE SPORICIDAL ACTION OF HYDROGEN PEROXIDE AND THE USE OF CRYSTALLINE CATALASE TO · DISSIPATE RESIDUAL PEROXIDE

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In a strict sense the germicidal activity of a chemical substance can be accurately evaluated only when the test organisms are quickly and completely freed from the toxic agent after the period of observation. With most germicidal substances this is not easily accomplished. The use of a neutralizing agent always introduces the uncertain influence of the added substance or its re-action products. The usual practice is to eliminate the toxic agent by sufficiently high dilution of the subculture. Since some compounds are bacteriostatic in extremely low concentration this technique is not always reliable.

Among the substances that possess strong germicidal activity, hydrogen peroxide is distinctive, in that the residual portion of the compound may be removed quickly and effectively from a solution after the desired period of reaction. This is made possible by the action of the enzyme catalase, which, under favorable conditions, rapidly decomposes hydrogen peroxide into water and molecular oxygen. Catalase-containing materials have been used in sterilization procedures involving peroxide, by Budde (1903), Böhme (1906), Much and Römer (1906), and others, but impurities associated with the catalase have materially limited their usefulness.

Important recent advances in enzyme chemistry have made it possible to prepare highly active catalase in crystalline form (Sumner and Dounce, 1937). Preparations possessing the purity and potency of crystalline catalase should, we believed, find appli-

cation in peroxide evaluation studies and also should contribute to the greater usefulness of hydrogen peroxide as a sterilizing agent. This paper presents the results of investigations designed to determine the validity of these assumptions.

METHODS AND MATERIALS

The test organisms were the spores of four aerobic species, *Bacillus albolactis, Bacillus cohaerens,* and two unidentified species chosen because of their resistance to heat.   The latter were obtained from the National Canners Association.   Culture No. 9499 was mesophilic; No. 1518 was an obligate thermophile.   The spores were cultivated and washed as previously described (Curran and Evans, 1937).   The stock suspensions of spores were stored at 6°C.   For testing purposes the requisite quantity of spores was uniformly suspended in 12 ml. of McIlvaine's buffer solution contained in a 50 ml. Erlenmyer flask.   The effervescence accompanying the decomposition of peroxide by catalase necessitates that a large air space be provided above the treated material. Except where otherwise indicated the test suspensions contained approximately 500,000 viable spores per milliliter and were buffered at pH 6.9.   Their thermal death times at 115°C. (heated in 1 ml. quantities in sealed tubes) were as follows: *B. albolactis,* 2½ minutes; *B. cohaerens,* 5 minutes; No. 9499, 12 minutes; and No. 1518, 2½ hours.   With one exception, the resistance of the spores remained practically unchanged during the course of the investigation.   The resistance of the spores of No. 9499 decreased materially after the beginning of the experiments, hence some discrepancies occur in the data recorded for this organism.

The peroxide used was Merck's Superoxol (approximately 30 per cent hydrogen peroxide).   The measurement of pH was made with the glass electrode (Beckman).   Catalase was prepared in crystalline form from beef liver according to the procedure outlined by Sumner and Dounce (1937).   The material was twice recrystallized, dissolved in a phosphate buffer (pH 7.0) and sterilized by Seitz filtration.   The purified sterile catalase was diluted with sterile distilled water to yield a solution containing 1.1 mg. of crystalline catalase (Kat. f. 28,000) per milliliter (Euler

and Josephson 1927).   This was used at the rate of 2 ml. to each 100 ml. of the test suspension at 30°C.   One milliliter was added immediately after the period required for the action of the peroxide, and another ml. was added ten minutes later.   Addition of catalase at intervals at the temperature indicated was found to provide favorable conditions for catalase activity.   At the end of one hour the suspensions were plated with glucose agar, and the plates incubated 48 hours at the optimum temperature of the organism.   The presence of hydrogen peroxide could not be detected in the test suspension at the time of plating.   The results presented are based on the average counts of triplicate plates and are representative of a large series of experiments in which close correspondence was consistently obtained.   The control spore suspensions subjected to the experimental conditions without hydrogen peroxide were not significantly affected.

EXPERIMENTAL

In figure 1 are curves showing the number of spores surviving different periods of exposure to 1 per cent hydrogen peroxide at 50°C. and at pH 6.9.   The logarithms of the number of survivors are plotted against time.   For each culture the experimental points fall essentially in a straight line, indicating that for most of the spores the death rate follows the law governing unimolecular reactions.   The values for k shown in figure 1 have been calculated from the following equation governing the rates of unimolecular reactions:

$$k = \frac{2.303}{t} \log \frac{No}{N}$$

where k = the constant, t = the time, No = the initial concentration of population, and N = the concentration of survivors at the time t.   For a small portion of the spores, comprising from 0.1 to 1 per cent of the total, the observed points deviated widely from the straight line; however, this does not preclude a unimolecular order of death for this fraction, since under the conditions of the experiment, considerable losses of peroxide occurred during the longer periods of exposure.

Influence of hydrogen-ion concentration

Since different concentrations of hydrogen ions affect oxidative changes differently, the effect of different levels of hydrogen-ion concentration upon the sporicidal action of hydrogen peroxide was determined. The periods of time required for the killing of 50 and 100 per cent of the spores at pH 3.0, 6.9, and 9.0 are shown in table 1. The period required for 50 per cent of the spores to be killed did not differ significantly among three of the four cultures.
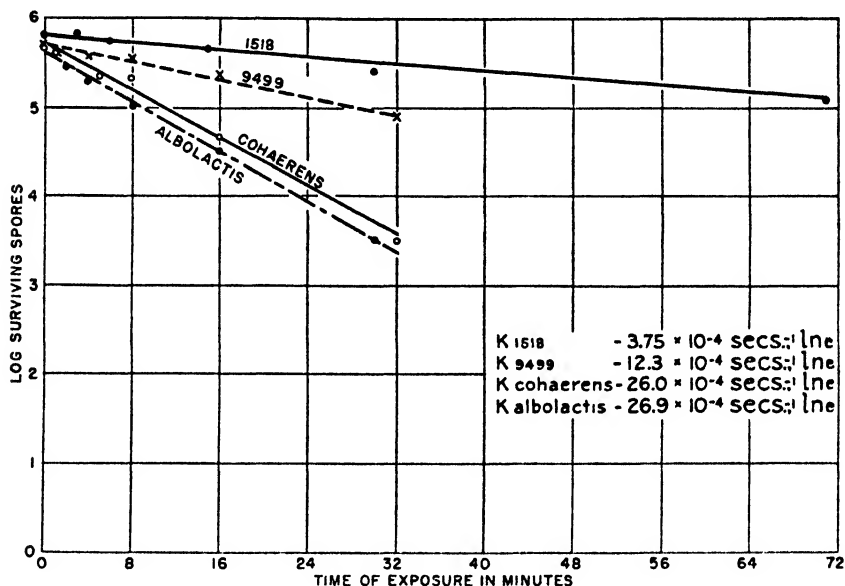
FIG. 1

Peroxide was much more destructive to the spores of No. 9499 in both acid and alkaline solutions than at pH 6.9. Reaction influences *per se* were not concerned with the differences recorded. The time required for total destruction of the spores increased as the pH was increased.

The influence of pH upon the stability of peroxide is a factor which must be considered in the interpretation of these data. The tendency of peroxide to decompose increases with hydroxyl-ion concentration. At pH 3.0 (50°C.) peroxide is very stable; at

pH 6.9 approximately 25 per cent is decomposed during the first 8 hours; at pH 9.0 about 75 per cent is dissipated during this period. Because of this fact, the apparently greater germicidal activity of peroxide at pH 3.0 over long periods is probably to be attributed to its greater stability at that pH than at pH 6.9 or 9.0 During the shorter periods required for the peroxide to destroy 50 per cent of the spores the reaction has no appreciable effect upon the concentration of peroxide.

As may be noted in table 1, at pH 6.9 and 9.0 some of the spores of culture No. 1518 survived the period during which the peroxide existed in effective concentration, a fact which is reflected in the persistence of viable spores after prolonged exposure.

TABLE 1

Influence of pH upon the sporicidal action of 1 per cent hydrogen peroxide at 50°C.

pH	B. ALBOLACTIS		B. COHAERENS		9499		1518*	
	50% reduction	100% reduction	50% reduction	100% reduction	50% reduction	100% reduction	50% reduction	100% reduction
	minutes	*hours*	*minutes*	*hours*	*minutes*	*hours*	*minutes*	
3.0	1 25	6.00	5.00	6.50	1.50	7 00	9.00	17.00 hours
6.0	1.75	7.50	5.00	6 50	5.00	8 25	10.00	7.00 days +
9.0	1.50	8.00	5.00	7.25	2.50	10 00	9 50	7 00 days +

* Concentration of spores 200,000 per milliliter.

Influence of temperature

As with most chemical reactions, the germ-killing activity of hydrogen peroxide is greatly influenced by the temperature at which it reacts. In table 2 is shown the time required for 1 per cent peroxide to kill 50 and 100 per cent of the spores through a 40-degree temperature range at pH 6.9. Each 10° rise in temperature decreased the 50 per cent destruction time from one-half to one-third. The time required to destroy all the spores usually decreased with rising temperature, but one exception may be noted. A shorter time was needed for total destruction of the spores at 30° than 40°C. At 30°C., under the conditions which prevailed, about 2.5 per cent of the peroxide is decomposed in 8 hours, while at 40°C. the decomposition is about 11 per cent.

This is probably responsible for the greater effectiveness of the peroxide at 30°C.   At 70°C. and pH 6.9 nearly all of the peroxide is decomposed in 5½ hours.   Above 40°C. the time required for total destruction of the spores decreased progressively with increases in temperature.   This may be explained on the assumption that the greater potency of the peroxide at higher temperatures more than compensates for the effect of losses of peroxide resulting from its decomposition.

TABLE 2

Influence of temperature upon the sporicidal action of 1 per cent hydrogen peroxide at pH 6.9

TEMPERA-TURE	B. ALBOLACTIS		B. COHAERENS		9499		1518*	
	50% reduction	100% reduction	50% reduction	100% reduction	50% reduction	100% reduction	50% reduction	100% reduction
°C.	*minutes*	*hours*	*minutes*	*hours*	*minutes*	*hours*	*minutes*	*days*
30	12.00	9.00	20.00	8.50	21.00	9.50	105.00	7.00+
40	6.00	12.00	10.00	10.00	10.00	12.00	40.00	7.00+
50	2.00	7.50	5.00	6.50	4.00	7.25	10.00	7.00+
60	1.00	7.00	2.50	5.25	2.00	5.50	3.50	7.00+
	seconds				*seconds*			
70	25 00	5.75	1.00	3.50	50.00	3.50	1.50	7.00+

* Concentration of spores 200,000 per milliliter.

Influence of concentration of spores and of peroxide

In table 3 is shown the influence of concentration of spores upon the time required for their complete destruction by 1 per cent peroxide.   This time increased more or less regularly as the number of spores in the suspension was increased.   It will be noted that the spores of culture No. 1518 were totally destroyed only in the 100,000 concentration.   Data for 50 per cent destruction are omitted in this table, since we were unable to obtain consistently close correspondence among separate experiments.

Table 4 shows the results obtained when the quantity of peroxide was increased through a narrow concentration range.   No significant relationships are apparent in these data.   By doubling the concentration of peroxide the time required for 50 per cent destruction was reduced more than one-half.   It is apparent from

these results that peroxide cannot be relied upon to produce very rapid or instantaneous killing of all the spores in a culture, even when used in high concentration. For most of the spores, a lag of variable length occurs between the addition of peroxide and measurable effects.

TABLE 3

Germicidal action of 1 per cent hydrogen peroxide at 50°C. upon spore suspensions of different density

CONCENTRATION OF SPORES	B. ALBOLACTIS	B. COHAERENS	9499	1518
	100% reduction	100% reduction	100% reduction	100% reduction
per ml.	hours	hours	hours	days
100,000	5.50	4 25	4.50	1.25
500,000	7.50	6.50	7.50	7.00+
2,500,000	9.00	8.25	10.75	7.00+
12,500,000	11.25	10 25	11.25	7.00+

TABLE 4

Sporicidal action of hydrogen peroxide at 50°C. as influenced by its concentration

CONCENTRATION OF HYDROGEN PEROXIDE	B. ALBOLACTIS		B. COHAERENS		9499		1518*	
	50% reduction	100% reduction	50% reduction	100% reduction	50% reduction	100% reduction	50% reduction	100% reduction
per cent	minutes	hours	minutes	hours	minutes	hours	minutes	days
1		7.50	5.00	6.50	4.00	7 25	10.00	7 00+
2		3.75	1 75	4 50	1.75	3 75	4 50	1.50
			seconds		seconds			
4		2 50	35.00	2 00	45 00	2 50	1 50	1 00

* Concentration of spores 200,000 per milliliter.

Peroxide-sterilized milk as a bacteriological medium

The rapidity and completeness with which hydrogen peroxide can be decomposed by crystalline catalase and the innocuousness of the decomposition products are factors that greatly increase the practicability of the use of hydrogen peroxide to produce effects of the sort discussed in this paper. Of interest in this connection is the possible utility of hydrogen peroxide in the sterilization of bacteriological media. To obtain information on this subject the growth of bacteria in milk sterilized by use of peroxide was

compared with that in milk sterilized by heat. In this experiment raw skimmed milk was heated with 1 per cent hydrogen peroxide at 55°C. for 2 hours, after which catalase was added as previously described. An equivalent quantity of catalase was added to the heat-sterilized sample before its inoculation to include the possible nutritional effect of this substance. The results obtained are shown in table 5. Of the four species used, *Escherichia coli, Pseudomonas aeruginosa,* and *Alcaligenes bookeri* grew as well in the peroxide-treated milk as in the heat control, although *A. bookeri* was slightly inhibited during the early hours

TABLE 5

Growth of bacteria in milk sterilized by hydrogen peroxide* and by heat†

PERIOD OF INCUBATION	PS. AERUGINOSA		E. COLI		ALC. BOOKERI		STREPT. LACTIS	
	H_2O_2 sterilized	Heat sterilized	H_2O_2 sterilized	Heat sterilized	H_2O_2 sterilized	Heat sterilized	H_2O_2 sterilized	Heat sterilized
hours								
0	14	18	11	12	4	4	60	65
6	2,600	3,000	161,000	176,000	130	300	1,530	3,490
24	880,000	710,000	1,370,000	1,290,000	137,000	108,000	156,000	804,000
48					102,000	106,000	200,000	450,000
Catalase production	Very abundant		Abundant		Slight		None	

* One per cent H_2O_2 at 55°C. for 2 hours.
† Autoclaved, 15 pounds for 15 minutes.
Inoculum = 0.05 ml. of an 18-hour milk culture.
Figures indicate number of bacteria per milliliter × 1,000.

of growth. The growth of *Streptococcus lactis* was markedly restrained throughout the 48 hours of observation.

These effects are of interest in connection with production of catalase by these organisms, roughly indicated in the lower part of table 5. *P. aeruginosa* and *E. coli*, which produce catalase profusely, show no evidence of being inhibited. *A. bookeri*, which produces smaller quantities of catalase, is inhibited in the early hours only, while *S. lactis*, which forms no catalase, is greatly retarded throughout the first 48 hours of growth. This suggests that traces of peroxide remained in the milk after treatment with catalase and were responsible for the retardation of the growth of

S. lactis. Our analyses showed, however, that if any peroxide were present, it was less than one part per million. That such concentrations could account for the observed retardation in growth of *S. lactis* seemed very improbable. Moreover, increas-

TABLE 6

Oxidation-reduction potentials of milk sterilized by hydrogen peroxide or by heat

PERIOD AFTER STERILIZATION	STERILIZED BY	
	Peroxide*	Heat†
hours	*Eh, volts*	*Eh, volts*
0	+0.3843	+0.2130
3	+0.3845	+0.2173
22	+0.3796	+0.2275

* One per cent peroxide at 55°C.—2 hours plus catalase.
† Autoclaved, 15 pounds for 15 minutes.
Temperature of determinations, 28°C.

TABLE 7

Influence upon the viability of spores suspended in milk of peroxide alone and of peroxide followed by heat

PERIOD OF EXPOSURE	9499		1518	
	70,000 spores per ml.		18,000 spores per ml.	
	H_2O_2* treatment at 55°C.	80°C. 15 minutes after H_2O_2 treatment	H_2O_2* treatment at 55°C.	80°C. 15 minutes after H_2O_2 treatment
hours				
0.5	+	+	+	+
1	+	+	+	+
1.50	+	−	+	+
2	−	−	+	−
2.50	−	−	−	−

+ = Some spores viable.
− = No spores viable.
* One per cent hydrogen peroxide.

ing the quantity of catalase used did not reduce the inhibiting factor for *S. lactis*. Table 6 shows the oxidation-reduction potentials of peroxide- and heat-treated milks at different periods after sterilization. Not only is the initial Eh much higher in the peroxide-treated sample, but in a surprising manner this positive

potential is very largely maintained during the first 22 hours. In view of this fact it seems probable that the retarded growth rate of *S. lactis* in milk sterilized by peroxide is the result of an unfavorable oxidation-reduction potential.

The appearance, taste, and odor of milk recently sterilized by hydrogen peroxide are only slightly inferior to those of pasteurized milk.   Upon long standing, however, it develops an objectionable oxidized flavor.   The intensity of this flavor seems to increase for a time and then to remain more or less unchanged for many months.   The flavor of peroxide-treated milk can be improved by heating it to 80°C. for 15 minutes after catalase action is complete.

The sporicidal effect of the combination of peroxide and heat (80°C., 15 minutes) is considerably greater than that of the peroxide alone.   This fact is brought out in table 7, which shows the results obtained when milk artifically inoculated with resistant spores was treated with 1 per cent hydrogen peroxide at 55°C. Two hours were required to sterilize the sample which contained 70,000 spores of culture No. 9499.   One and one-half hours were sufficient when the sample was heated to 80°C. for 15 minutes after peroxide treatment.   The sample inoculated with the more resistant No. 1518 spores required a somewhat longer time with only 18,000 spores per milliliter, and this time also could be shortened by combining heat with peroxide treatment.

DISCUSSION

Temperature, pH, concentration of reagent, and concentration of exposed organisms are important factors influencing the sporicidal activity of hydrogen peroxide.   In approximately neutral solutions the sporicidal action of peroxide tends to increase with rising temperatures.   An exception to this rule has been noted. The influence of pH seems to be closely correlated with its effect upon the stability of the peroxide, hence in acid solutions the sporicidal activity of peroxide is enhanced while alkaline reactions which promote its decomposition tend to reduce the germicidal activity of peroxide.   Organic matter, apart from its possible catalase content, seems to have comparatively little influence upon the germicidal action of hydrogen peroxide.   Our

observations indicate that suspensions of spores in catalase-free broth, and milk can be sterilized almost as readily by peroxide as can water and buffer suspensions of comparable pH. This is a factor which is often of considerable importance in the selection of a germicide.

SUMMARY

The evaluation of the germicidal activity of hydrogen peroxide is greatly facilitated by the use of purified (crystalline) catalase which affords a rapid and efficacious means of dissipating residual peroxide.

The logarithmic curve for survival of spores exposed to 1 per cent hydrogen peroxide at 50°C. (pH 6.9) indicates that the rate of killing of most, and possibly all, of the spores is described by the unimolecular law for chemical reactions.

Hydrogen-ion concentration affects the apparent sporicidal activity of hydrogen peroxide, largely through its influence upon peroxide stability. Thus, there is greater sporicidal activity in acid media than in neutral media and usually less in strongly alkaline than in neutral media.

In the 40° to 70°C. (pH 6.9) range the time required for the destruction of 50 per cent of the spores by 1 per cent hydrogen peroxide decreases from one-half to one-third for each 10° rise in temperature. The time required for total destruction of the spores through this range usually decreases with each 10° rise in temperature. The spores of some species cannot be totally destroyed by this treatment through this range of temperature.

As the temperature is raised from 30° to 40°C. the time required for the destruction of 50 per cent of the spores decreases from one-half to one-third. The time required for total destruction of the spores increases with this change in temperature.

The time required for the destruction of spores by hydrogen peroxide increases as the concentration of spores is increased, and decreases as the concentration of peroxide is increased. Complete destruction of the spores in a culture requires appreciable time— even when high concentrations of peroxide are used.

Milk sterilized by hydrogen peroxide supports the growth of

Escherichia coli, Pseudomonas aeruginosa, and *Alcaligenes bookeri* as well as milk sterilized by heat.   Milk sterilized by hydrogen peroxide retards the growth of *Streptococcus lactis;* this is probably caused by the high oxidation-reduction potential of milk treated with peroxide.

The appearance and odor of milk sterilized by peroxide are essentially the same as those of raw milk; its flavor initially is only slightly inferior to raw milk if the treated milk is heated to 80°C. for 15 minutes immediately after peroxide treatment.   Upon long standing, milk treated with peroxide develops an objectionable oxidized flavor.

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THE DEMONSTRATION OF PHOSPHATASE IN NEISSERIA[1]

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Although the study of bacterial enzymes has received much attention during the last few years, it was not until recently that the presence of phosphatase in microörganisms was established. The first evidence that this enzyme occurred among bacteria was the demonstration of the ability of *Escherichia coli* to hydrolyze hexosephosphate (Manning, 1927). Several years later, Boivin and Mesrobeanu (1933) observed that inorganic phosphate was liberated from a variety of other substrates on hydrolysis by this organism at characteristic pH optima. In 1938, Pett and Wynne measured the activity of phosphatase in a heterogeneous group of saprophytic bacteria and noted, in addition, an optimal pH for each organism. Pett (1939) suggested that the presence of phosphatase might prove valuable in the classification of bacteria.

A recent study by Leahy, Sandholzer and Woodside (1939) showed that, of a considerable number of strains of gram-negative bacilli belonging to the family *Enterobacteriaceae*, every one tested hydrolyzed disodium-phenyl-phosphate. By employing this substrate, quantitative information was obtained with the use of smaller quantities of bacterial cells than had heretofore been possible. The optimal pH varied among the different genera and even among strains of the same species. The present report, a continuation of this study, deals with the demonstration of

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phosphatase in the *Neisseria*, especially in *Neisseria gonorrhoeae*. It was undertaken with the hope that the new approach offered by quantitative biochemical methods might yield data pertinent to a better understanding of the relationships in this particular group where other methods of approach have not been very fruitful.

METHODS

Preparation of the bacterial cells

For the present investigation, 16 strains of *Neisseria* were employed. Of these strains, 10 were *Neisseria gonorrhoeae*, 2 *Neisseria intracellularis*, 2 *Neisseria catarrhalis*, and 2 *Neisseria sicca*. With the exception of preparations GN I and GN II, each strain was grown for 6 or 7 days at 37°C. in Douglas's broth containing 5 per cent of ascitic fluid. The cells were removed by centrifugation, washed once in a 0.85 per cent solution of NaCl (physiological saline solution), "lyophilized" by the technic of Flosdorf and Mudd (1935), placed in stoppered test tubes, and stored at 5°C. until measurements were made. The preparations of dried cells from strains GN I and GN II were obtained from 6-day-old veal-infusion-broth cultures.[2]

Preparation of the substrate solutions

The buffered substrate solutions employed for the measurement of pH optima were prepared as described by Leahy, Sandholzer and Woodside (1939). The 0.005 M disodium-phenyl-phosphate was buffered with a 0.05 M phthalate or veronal buffer at each pH unit from 4.0 to 9.0. After adjustment to the desired pH value had been made, it was checked by the glass-electrode method before the solution was used.

Measurement of optimal pH

The phosphatase activity of the "lyophilized" organisms was measured by the method of Leahy, Sandholzer and Woodside (1939). In brief, the procedure consisted in adding a known

[2] Supplied by Parke, Davis and Company, Detroit, Michigan.

weight (from 2 to 5 mgm.) of "lyophilized" cells suspended in distilled water to a series of tubes containing buffered substrate solutions at each pH unit from 4.0 to 9.0.   The mixtures were incubated for 24 hours at 37°C., ±0.02°C., and the degree of hydrolysis was measured from the amount of phenol produced. Quantitative colorimetric estimation of the phenol was made by the use of Gibbs's reagent (2,6-dibromoquinonechloroimide), which is capable of measuring 0.1 gamma of phenol in 10 ml. The activity of the various preparations was expressed in gammas of phenol liberated per milligram of "lyophilized" cells in 24 hours at 37°C. after a small correction, as indicated by the controls, had been made.   In every instance, the pH of maximal activity was determined by plotting curves of the phenol values at each pH.   Duplicate runs with different preparations of the same strain, and repetition of measurements, both in the absence and in the presence of magnesium ions, gave optima agreeing within ±0.2 pH unit.

RESULTS

The optimal pH values for the organisms of the genus *Neisseria* selected for study, are shown in table 1.   Without exception, all of the strains examined possessed phosphatase activity.   The maximal activity of the different preparations varied widely. Two strains of *Neisseria gonorrhoeae*, 21 and 24, were the most active of all the *Neisseria* examined, while the 2 strains of *Neisseria sicca* displayed the weakest activity.

Many of the strains manifested sharp pH optima, but these optima varied considerably among the several preparations measured.   Of the 16 strains of *Neisseria*, 5 strains of *Neisseria gonorrhoeae* had sharp optima within a narrow range from pH 4.7 to 4.9, 9 from pH 6.1 to 6.4, and 1 at pH 5.2.   On the other hand, another strain of *Neisseria gonorrhoeae* possessed a comparatively broad pH range of activity, from pH 6.8 to 7.4.

An experiment to determine the effect of storage upon the activity of a single preparation (GN I) is illustrated in figure 1. Storage for a period of 100 days at 15 mm. pressure at room temperature decreased the activity by 32 per cent.   Another ex-

TABLE 1

The maximal activity of phosphatase at optimal pH in various strains of Neisseria

STRAIN NUMBER	ORGANISM	SOURCE	MAXIMAL ACTIVITY AT OPTIMAL pH	pH OF MAXIMAL ACTIVITY
			γ phenol per mgm. cells	
6	*N. gonorrhoeae*	Joint	35.3	4.7
10A	*N. gonorrhoeae*	Urine	50.3	4.8
16A	*N. gonorrhoeae*	Urine	40.2	4.8
2	*N. gonorrhoeae*	Eye	30.8	4.9
16	*N. gonorrhoeae*	Urine	43.0	4.9
24	*N. gonorrhoeae*	Cervix	60.5	5.2
21	*N. gonorrhoeae*	Joint	61.3	6.1
GN I	*N. gonorrhoeae*	Urethra	41.2	6.1
69	*N. gonorrhoeae*	Urine	29.5	6.3
GN II	*N. gonorrhoeae*	Urethra	47.6	7.2*
38	*N. intracellularis*	Sp. fluid	14.6	6.2
18	*N. intracellularis*	Sp. fluid	58.0	6.4
60	*N. catarrhalis*	Urine	21.3	6.5
26	*N. catarrhalis*	Urine	21.0	6.7
34	*N. sicca*	Urine	12.7	6.1
41	*N. sicca*	Urine	12.9	6.2

* The range of optimal pH was from pH 6.8 to 7.4.
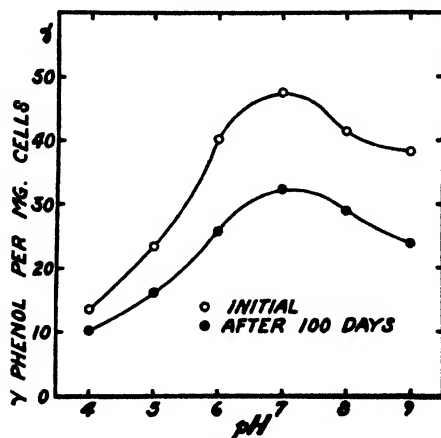
FIG. 1. pH-ACTIVITY CURVES OF NEISSERIA GONORRHOEAE (GNII) BEFORE AND AFTER STORAGE FOR 100 DAYS AT 15 MM. PRESSURE AT ROOM TEMPERATURE

periment to determine the effect of magnesium ions upon the same preparation (fig. 2) showed that a 0.001 M concentration of these ions stimulated the activity by 11 per cent. Strikingly, in each experiment, the pH of maximal activity remained unchanged.
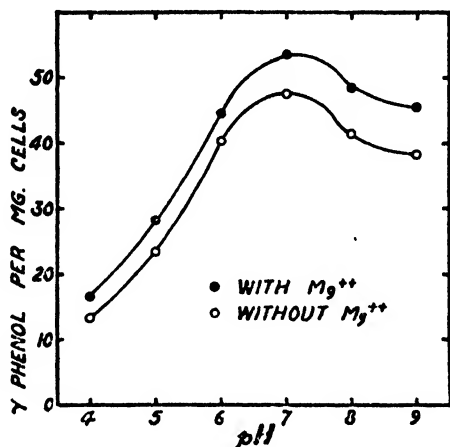
FIG. 2. pH-ACTIVITY CURVES OF NEISSERIA GONORRHOEAE (GNII) WITH AND WITHOUT ADDED 0.001 M. MAGNESIUM IONS

DISCUSSION

The use of a sensitive substrate (disodium-phenyl-phosphate) and the controlling of conditions affecting hydrolysis have made it possible to measure the activity of phosphatase by a method in which the test-organism was the only variable. Application of this method to the genus *Neisseria* disclosed that both the optimal pH and the maximal activity varied markedly among the different organisms examined. Of these characteristics, the variation of the optimal pH is considered to be the more significant for the following reasons. First, although repeated measurements on duplicate preparations have shown a decreased activity upon storage (fig. 1), the pH of maximal activity remained constant. Second, measurements made in the presence of added magnesium ions (fig. 2) have shown an increased activity, while again the optimal pH failed to show a significant change.

The 5 strains of *Neisseria gonorrhoeae* which had optima in the range of pH 4.7 to 4.9 possessed the most acid optima of all the microörganisms yet encountered in our studies using disodium-phenyl-phosphate. The *Neisseria* as a whole, furthermore, were found to have maximal activities far below those of the *Enterobacteriaceae*, as described in a previous study.

SUMMARY

Phosphatase has been demonstrated for the first time in *Neisseria gonorrhoeae*, *Neisseria intracellularis*, *Neisseria catarrhalis*, and *Neisseria sicca*. The optimal activity occurred at pH values ranging from pH 4.7 to 7.2. Each strain possessed a single characteristic optimum.

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SEROLOGICAL TYPES OF CLOSTRIDIUM PERFRINGENS

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In the flood of papers on the pathogenic anaerobes which appeared about 1920 several workers, particularly Zeissler (1919), Heller (1920), Hall (1922), came to the conclusion that agglutination reactions were of no value in the classification of the group. The apparent heterogenicity of strains of *Clostridium perfringens* has probably caused the greatest difficulty. Weinberg and Barotte (1928), Howard (1928), Wilsdon (1931), Ris (1936) and others have produced agglutinating sera for this species in relatively high titre notwithstanding Robertson's (1928) apparent failure; yet in their review of the serological relationship of anaerobes McCoy and McClung (1938) remark: there is "nothing but confusion" concerning the agglutination reactions of *C. perfringens.*

The extremes of opinion concerning the antigenic relationship among members of this species may be represented by the work of Wilsdon (1931) and Meisel (1938). Wilsdon found that his strains were agglutinated only by homologous sera. However, he included both *C. perfringens* of human origin and also those of the rather confusing group isolated from disease of herbivorous animals, particularly sheep, referred to as *Bacillus agni, Bacillus plaudis, Bacillus ovitoxicus.* It will be remembered that these organisms are distinguished from each other and from the classical *C. perfringens* largely on the specificity of the toxins they produce. Whether these organisms should be included in the *C. perfringens* species remains to be determined. On the other hand Meisel found that six of ten cultures of Type A *C. perfringens* examined belong to one serological group and that four are serologically heterogeneous.

In the course of other studies on *C. perfringens* we have had occasion to examine the agglutination reactions of some eighty-five cultures from a variety of sources and precipitin reactions of a part of the group. Some seventy-five of these cultures belong to Wilsdon's (1931) toxogenic Type A. The balance failed to produce a measurable amount of toxin and therefore could not be typed.

AGGLUTINATION REACTIONS

No difficulty was experienced in obtaining relatively high-titre serum in rabbits with all strains tested. The procedure consisted in injecting four doses of heat-killed organisms from 16-hour broth cultures, followed by four to eight doses of living 16-hour cultures. The doses were given intravenously daily for four days, followed by four days' rest, and the procedure repeated once or twice. The 16-hour cultures yielded abundant growth but a relatively small amount of toxin; moreover, spores do not form in this period so that 65° for one hour was ordinarily sufficient to sterilize. Similar 16-hour cultures in broth were used in preparing antigens for agglutination reactions. The cultures were diluted with broth to the same opacity and neutral formalin added to make 0.5 per cent. Similar results were obtained with or without the formalin.

The results of agglutination reactions between 16 antisera and 85 antigens prepared from cultures from a variety of sources, including those homologous with the antisera, are shown in table 1. It is apparent that the first six cultures, shown in the table, constitute a group with a similar antigenic structure, in sharp contrast with the remaining 79 cultures listed in the table. The extent of the antigenic relationship among these 6 cultures is shown more precisely by the agglutinin adsorption results, table 2. Five of the 6 cultures, SW, WX, 29, Q2 and Q3, are antigenically indistinguishable, by the procedure used, except for the extent to which they exhibit antigens common with cultures 28 and R1. The relationship of culture 28 to the group is more complex. It is evident that this organism is antigenically related to four members of the group but not to the fifth, WX.

TABLE 1

Agglutination reactions between antisera for 16 cultures C. perfringens and antigens from 85 cultures

ANTIGENS	SW	WX	29	28	R1	R5	C4	I	II	III	IV	LS	104	54	74	36	SOURCE OF CULTURE
SW	+++	+++	+++	−	−	−	−	−	−	−	−	−	−	−	−	−	Horse manure
WX	+++	+++	+++	−	−	−	−	−	−	−	−	−	−	−	−	−	Horse manure
29	+++	++	+++	−	−	−	−	−	−	−	−	−	−	−	−	−	Gas gangrene
Q2	+++	++	+++	−	−	−	−	−	−	−	−	−	−	−	−	−	Normal human faeces
Q3	+++	++	+++	−	−	−	−	−	−	−	−	−	−	−	−	−	Normal human faeces
28	+	−	−	+	+++	+	++	+	−	−	−	−	−	−	−	−	Gas gangrene
R1	−	−	−	+	+++	+	++	+	−	−	−	−	−	−	−	−	Cow urine
R5	−	−	−	−	++	++	−	−	−	−	−	−	−	−	−	−	Cow urine
C4	−	−	−	+	+++	−	+	−	++	−	−	−	−	−	−	−	Gas gangrene
I	−	−	−	+	++	+	++	+	++	+	−	−	−	−	−	−	A.T.C.C.
II	−	−	−	−	++	−	++	+	++	+	−	−	−	−	−	−	A.T.C.C.
III	−	−	−	−	−	−	−	+	+	++	+	−	−	−	−	−	A.T.C.C.
IV	−	−	++	−	−	−	−	−	−	−	++	−	−	−	−	−	A.T.C.C.
LS	−	−	−	−	−	+	−	−	−	−	+	++	+	−	−	−	Gas gangrene
104	−	−	−	−	−	−	−	−	−	−	−	++	+	−	−	−	Gas gangrene
54	−	−	−	−	−	−	−	−	−	−	−	+	−	++	+	−	Gas gangrene
74	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+++	+	Gas gangrene
36	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+++	Gas gangrene
22 cultures from gas gangrene	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	
30 cultures from normal human faeces	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	Normal human faeces
15 cultures from soil or manure	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	Soil or manure

+++, agglutination in 1:640 or greater dilution of the serum; ++, agglutination in 1:160 to 1:320 dilution of the serum; +, agglutination in 1:20 to 1:80 dilution of the serum; −, no agglutination.

It is also apparent that culture 28 differs from the other members of this group in that it possesses antigenic components in common with several other cultures. The 6 antigenically related cultures were isolated, as shown in table 1, from the following sources: 2 from gas gangrene, 2 from horse manure, 2 from normal human faeces.

Beyond this subgroup, it is apparent from tables 1 and 3, that there is no opportunity, with the data in hand, of making

TABLE 2

Adsorption of agglutinins from four samples of C. perfringens antisera by antigens from six cultures

ANTISERA	PER CENT AGGLUTININS ADSORBED BY					
	SW	WX	29	Q2	Q3	28
SW	100	100	100	80	80	60
WX	100	100	100	80	80	0
29	100	100	100	80	80	60
28	10	0	10	0	0	100

TABLE 3

Adsorption of agglutinins from four samples of C. perfringens antisera by antigens from four cultures

ANTISERA	PER CENT AGGLUTININS ADSORBED BY			
	I	II	III	104
I	100	0	0	0
II	10	100	10	0
III	0	0	100	0
104	0	0	0	100

any further groupings. It is equally evident, however, that there is some antigenic overlapping. This is most apparent in the case of cultures R1, C4, I and IV. It is quite possible that, with the examination of a much larger number of cultures, it might be possible to divide the species into antigenic groups but, if it is impossible to do so with 85 cultures from a considerable variety of sources, it is unlikely that such a hypothetical grouping would have any significance.

The possibility that the heterogenicity is the result of loss of specific agglutinogen through variation was tested in two ways on one culture, WX. Several cultures of this strain, one or more years old, were plated and fresh cultures prepared from a number of single colonies. All were agglutinated with the specific serum at the same titre. Several of these were carried through twenty daily serial transfers, plated and cultures prepared from single colonies. All were agglutinated with the specific serum at very nearly the same titre.

Even S to R variation, as described by Orr, Josephson, Baker and Reed (1933), did not result in complete loss in specific agglutinogen. Anti-S serum agglutinated both S and R organisms isolated from the same culture and, conversely, anti-R serum agglutinated both S and R organisms from the same culture. Such a comparison was only possible in those instances where R organisms formed sufficiently stable suspensions to permit carrying out the reaction.

PRECIPITIN REACTIONS

Several methods of preparing antigens for precipitin reactions were tested. Best results were obtained with acid-extracts of 18 hour cultures prepared according to the method used by Lancefield, (1925, 1928) with streptococci. Such acid extracts, in dilutions up to 1:200, gave good precipitin reactions with homologous serum (as used in the agglutination reactions). Higher dilutions of the antigen generally failed to react with undiluted or diluted serum.

Results with 9 antisera and acid-extracts from 24 of the cultures used in agglutination reactions are shown in table 4. Comparison of these results with the agglutination reactions, table 1, leads to the same conclusions as to the antigenic structure of the several cultures. It is again apparent that SW, WX, 29 and 28 constitute a related group of cultures, with culture 28 in a less clearly defined position. (Two additional cultures shown by agglutination to belong to this group were not included in the precipitin reactions.) The remaining cultures are, by these reactions, unrelated or show only slightly overlapping antigenic components.

Though the results from agglutination and precipitin analysis are not identical, the difference appears too slight to justify more extensive examination.

The rather unsupported statements of Jiminez (1936) and Meisel (1936) that specific polysaccharides may be isolated from *C. perfringens* and other anaerobes lend some support to the probability that the specific substance contained in acid-extracts of the various cultures of *C. perfringens* is a polysaccharide.   To

TABLE 4

Precipitin reactions between antisera for nine cultures C. perfringens and acid-extract antigens from twenty-four cultures

ANTIGENS, ACID EXTRACTS	ANTISERA								
	SW	WX	29	28	C4	II	III	IV	LS
SW	+++	+++	+++	+	−	−	−	−	−
WX	+++	+++	+++	+	−	+	+	−	−
29	+++	+++	+++	+	−	+	+	−	−
28	−	+	−	++	+	+	−	−	−
C4	−	−	−	−	+++	−	−	−	−
II	−	+	−	+	−	+++	+	−	+
III	−	+	−	−	−	+	+++	−	−
IV	−	−	−	−	−	+	−	+++	−
LS	−	+	−	+	−	−	−	−	+++
R1	+	+	+	−	−	−	−	−	−
14 cultures from various sources	−	−	−	−	−	−	−	−	−

+++, precipitate with 1:100 dilution of antigen; ++, precipitate with 1:10 dilution of antigen; +, precipitate with undiluted antigen; −, no precipitate with undiluted antigen.

determine this, a concentration and partial purification was made using a modification of the familiar alcohol precipitation procedure of Heidelberger and Avery adopted from Lancefield's (1925) procedure of obtaining soluble specific substance from *Streptococcus viridans*.   A large volume of 18 hour culture in broth of C4 *C. perfringens* was evaporated to a small volume, precipitated with acetic acid and later by boiling.   The supernatant was then repeatedly precipitated with two volumes of alcohol.   The final precipitate gave the usual chemical tests

for polysaccharide and failed to give protein reactions. It gave precipitin reactions, in dilutions to 1:2000, with the homologous antiserum but failed to react with heterologous *C. perfringens* sera.

Four cultures belonging to Wilsdon's (1931) toxin Types C and D were not agglutinated by any of the antisera listed in table 1, nor did acid-extracts from the four cultures show any precipitin action with the sera listed in table 4.

<div style="text-align:center">CONCLUSIONS</div>

It is shown that in a group of 85 cultures of *Clostridium perfringens*, most or all of which belong to Wilsdon's toxigenic Type A, six show a close antigenic relationship on the basis of agglutination and precipitin reactions. A few others show some overlapping of antigenic components and the balance, by these methods of analysis, show no antigenic relationship. Earlier results which have shown no antigenic similarity between cultures, or results such as those of Meisel (1938) which indicate 6 out of 10 cultures to be antigenically similar, have probably given an incorrect picture of the antigenic heterogenicity or homogenicity of the species as a result of the analysis of too few cultures. Analysis of a much larger group of cultures might make possible arrangement of the species in antigenic groups but from the present results it is evident that the number of groups would be very large.

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A COMPARISON OF COBALT AND NICKEL SALTS WITH OTHER AGENTS FOR THE DETECTION OF HYDROGEN SULFIDE IN BACTERIAL CULTURES

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INTRODUCTION

Numerous methods have been proposed for the detection of hydrogen sulfide production by bacteria, several having been developed in recent years. Among investigations in which this reaction has been used as a means of differentiating bacteria are the contributions of Jordan and Victorson (1917) on the paratyphoid-enteritidis group, Kligler (1917) concerning the typhoid-paratyphoid group and Levine and co-workers (1932, 1934 and 1936) involving the colon-aerogenes group. Comparisons of various methods with larger groups of bacteria have likewise been made by ZoBell and Feltham (1934) and Tittsler and Sandholzer (1937) who compared lead and iron salts. Hunter and Creselius (1938) introduced bismuth sulfite for the purpose of comparing it with iron.

A survey of the literature revealed two facts. First, with one exception, the use of cobalt and nickel salts as detecting agents for hydrogen sulfide has not been reported. The exception is found in a report by Sacquépée and Chevrel (1905) who found that nickel sulfate gave positive hydrogen sulfide tests with cultures of *Salmonella schottmülleri* but not with those of *Salmonella paratyphi*. Second, a systematic comparison of media containing these various detecting ions has never been attempted with a representative group of bacteria.

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Certain disadvantages exist with all of the indicators now in use for the detection of hydrogen sulfide. Iron salts tend to hydrolyze and precipitate from solution. Neither are they as sensitive in neutral or acid media as other detecting ions whose sulfides have a lower solubility product. Lead and bismuth are limited in their use because of their toxicity. Compounds of bismuth are difficult to keep in solution. Theoretically cobalt and nickel are somewhat more sensitive to hydrogen sulfide than iron and nearly as sensitive as bismuth. In addition, they possess the added advantage that their salts are quite soluble and do not hydrolyze in alkaline or neutral media as do iron and· bismuth salts. Thus, cobalt and nickel ions possess the requisite qualities of solubility, sensitivity and distinct color change desirable for hydrogen sulfide detection. However, all metallic ions are subject to the limitation that they attain maximal sensitivity for sulfide ion only at higher pH levels.

Since previous investigations have indicated that hydrogen sulfide detection may vary with the method used, the object of the following studies has been to compare some of the more recent methods with one employing cobalt and nickel as detectors.

<div align="center">EXPERIMENTAL</div>

<div align="center">*A. Toxicities of cobalt and nickel ions for bacteria*</div>

Before developing a medium containing cobalt and nickel, it was necessary to determine the tolerance of the test organisms to various concentrations of salts of these metals. The bacteria selected represented a number of genera predominantly of the Enterobacteriaceae group and were species capable of growing aerobically upon nutrient agar. A number of the pathogenic species used were obtained from the stock culture collection of the Kansas State Board of Health Laboratories.

Hotchkiss (1923) studied the effects of varying concentrations of solutions of a number of metallic salts, including cobalt and nickel, on *Escherichia coli* (*communis*). With these and other metals, the medium (metal salt in Bacto peptone) could not be heat-sterilized and still yield consistent results, for the salts either

hydrolyzed or formed precipitates with the peptone. A procedure was therefore devised which eliminated these undesirable conditions.

Nutrient broth and 0.0050 M solutions of cobalt and nickel nitrate hexahydrates were prepared separately. Measured quantities of the broth were then placed in filter flasks on which were mounted Berkefeld filters. After the assembly (flasks, broth and filters) had been sterilized by autoclaving at 15 pounds for 20 minutes, measured quantities of the salt solutions were filtered into the broth until the desired metal ion concentrations had been attained. The filter candles were then replaced by sterile tubing bells (Riker and Riker, 1936), the flasks inverted (the side-arm tubes then served as a means of admitting air to the flasks, thus maintaining atmospheric pressure within) and the contents aseptically distributed in sterile test tubes. The final concentrations of both nickel and cobalt salts in nutrient broth were 0.0010 M, 0.00050 M, 0.00010 M, and 0.000050 M. These were inoculated with single loop transfers of twenty-four hour broth cultures. Turbidity in the broth tubes was used as a criterion of growth. Incubation was at the optimum growth temperature for the individual organisms concerned. After twenty-four hours it was found that every test organism except *Bacillus kaustophilus* had grown to a visible extent in the two weaker solutions. In some instances, with further incubation, growth was apparent in the concentrated solutions, but for the majority of the organisms 0.00010 M cobalt remained the highest level tolerated. Identical tests employing nickel demonstrated that, in general, the bacteria could tolerate nickel solutions of 0.00050 M and lower concentration. Thus, the tolerated concentration of nickel nitrate was about five times that of cobalt nitrate. In subsequent work, therefore, 0.00010 M cobalt and 0.00050 M nickel solutions were employed. Only five of the test species would not grow in the 0.00050 M nickel solution; these were *Mycobacterium phlei, Sarcina lutea, Rhodospirillum rubrum, Bacillus kaustophilus,* and *Klebsiella friedländerii.* These organisms, for which hydrogen sulfide production has never been observed, were omitted from further tests.

B. Preparation of the cobalt-nickel medium

Factors influencing the detection of hydrogen sulfide in a medium incorporating cobalt and nickel ions were systematically studied. A number of media were prepared, each differing in one variable and the effect upon the test was noted. These experiments indicated the following optimum conditions.

Sulfur sources. The naturally-occurring sulfur compounds found in the peptone constituents of the media were not sufficient for the detectable production of hydrogen sulfide; hence an additional sulfur source was necessary. Among those investigated were one organic and two inorganic compounds. A combination was also employed. Sodium sulfite proved unsatisfactory. Cysteine yielded much better tests than did sodium thiosulfate, the third sulfur source tested. All organisms producing hydrogen sulfide at all were always able to yield that product from cysteine. The latter was more desirable than a cysteine-sodium thiosulfate mixture.

Glucose. The addition of one gram of glucose per liter of medium resulted in better tests for hydrogen sulfide than when it was omitted.

Hydrogen ion concentration. Two pH levels, both slightly on the alkaline side, were selected because metal ions are theoretically more sensitive at higher pH values. A pH of 7.1 was the preferred one for the detection of hydrogen sulfide for all microorganisms except *Proteus vulgaris*, which produced it in amounts detectable by this test at pH 7.8 but not at the former pH level although it may be formed at the lower level also. Tarr (1934) reported similar behavior of this organism.

Scant or negligible hydrogen sulfide production, apparent in most instances at the higher pH, is perhaps due to the inhibitory action of that hydrogen ion concentration upon the metabolism of the bacteria.

Protein source. Bacto-proteose peptone, one of the two nitrogen sources used, produced somewhat better results than did Bacto-tryptone.

Hydrogen sulfide indicator. Cobalt was more sensitive than

nickel, as an indicator of hydrogen sulfide, in that it yielded positive tests in a larger number of cases.   This is to be expected in view of the lower solubility product.   However, nickel gave better tests than did cobalt (as evidenced by more intense darkening of the media) in the instances when it did give positive tests. Despite the fact that the concentrations of the two individual metals were near the maximum tolerated values, it was found that when those two concentrations of nickel and cobalt nitrates were employed in one medium, no cumulative inhibitory action took place.   The combination gave tests much superior to those obtained when either of the two metals was used singly.

The medium regarded as the best combination of the different variable factors investigated, and discussed above, has the following composition:

Proteose peptone (Difco)	20.0 grams
K₂HPO₄..	1.0 gram
Glucose	1.0 gram
Cysteine hydrochloride (Merck)	2.0 grams
Agar (Bacto)	15.0 grams
0.0050 M Co(NO₃)₂ solution* .	20.0 ml.
0.0050 M Ni (NO₃)₂ solution† .	100 0 ml.
Water	880.0 ml.

* 0.727 grams $Co(NO_3)_2 \cdot 6H_2O$ in 500 ml. water.
† 0.728 grams $Ni(NO_3)_2 \cdot 6H_2O$ in 500 ml. water.

The medium was adjusted to a pH of 7.2 ± 0.1 with N/1 NaOH, then tubed and sterilized at 15 pounds for 20 minutes.   The nickel and cobalt solutions can be made up as one solution if desired.

COMPARISON OF VARIOUS MEDIA FOR THE ABILITY TO DETECT THE PRESENCE OF HYDROGEN SULFIDE

The cobalt-nickel medium discussed in the preceding paragraph (designated as medium E in the table of results) was compared with four other media used for hydrogen sulfide detection. They were Bacto-Lead Acetate Agar (A), Bacto Iron Peptone Agar (B), Bacto-Kligler Iron Agar (C), and Hunter's bismuth sulfite tryptone agar with skim milk (D) (Medium III, Hunter

and Crecelius, 1938).   These media were prepared, tubed, and sterilized simultaneously.   In these tests, as in all preceding ones,

TABLE 1

ORGANISM	NUMBER OF STRAINS	MEDIUM				
		A	B	C	D	E
Aerobacter aerogenes.......	6	−	−	−	+	+++
Aerobacter cloacae.........	1	−	−	−	−	++
Aerobacter oxytocum.......	1	−	++	+	+	+++
Aerobacter viscosum.......	1	−	++	+	−	++
Alcaligenes viscosus.......	1	−	++	+	+	++
Escherichia coli...........	7	−	−	−	+	++
Eberthella typhosa....... ..	4	++	++	+++	+	−
Shigella dysenteriae.......	2 {	(1) −	−	−	+	−
		(2) −	−	−	−	−
Shigella paradysenteriae (Flexner).......	4 {	(1, 2, 3) −	−	−	+	−
		(4) −	−	−	−	−
Shigella sonnei........ ...	3 {	(1) −	−	−	+	−
		(2) +	−	−	+	+
		(3) −	−	−	+	−
Shigella sp. (Newcastle)..	1	−	−	−	+	−
Salmonella gallinarum.....	4	+	−	+	+	++½
Salmonella paratyphi......	3	−	−	−	+	+½
Salmonella schottmuelleri......................	4	++	++½	+++	+½	++½
Salmonella pullorum......	1	+½	+½	+½	+	−
Salmonella enteritidis.....	1	−	−	−	+	++
Salmonella typhimurium..	1	++	+++	+++½	+	+++
Proteus vulgaris...........	4 {	(1, 2) −	−	−	−	−
		(3, 4) ++	++	+++	+½	−
						++ if pH is 7.8
Slow lactose fermenter....	2	++	+++	+++	+ weak	+++
Proteus morgani..........	1	+	−	−	−	+½
Serratia marcescens.......	1	−	−	−	+ weak	−
Streptococcus lactis.......	1	−	−	−	+	−
Citrobacter sulfidogenes....	1	++½	+++	++½	−	+++

single stab inoculations were made from twenty-four hour nutrient broth cultures.   Observations were made at the end of 1, 2, 3 and 5 day intervals, following incubation at 37°C.

RESULTS

A positive reaction for hydrogen sulfide consisted of a general darkening of the medium, or of a definite localized browning or blackening along the line of puncture. The organisms which yielded positive tests are listed in Table 1, together with estimations of the relative response in the various media.

The following organisms did not give evidence of hydrogen sulfide production under any of the experimental conditions: *Alcaligenes fecalis*, *Neisseria catarrhalis*, *Leuconostoc mesenteroides*, *Brucella abortus*, *Staphylococcus albus*, *Staphylococcus aureus*, *Micrococcus tetragenus*, *Flavobacterium vitarumen*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Bacillus megatherium*, *Alcaligenes radiobacter*, and *Corynebacterium xerosis*.

DISCUSSION

The following facts, gathered from the above table, are apparent. The lead acetate, iron peptone, and Kligler iron media gave many positive tests; however, they were not quite as sensitive as the bismuth and the cobalt-nickel media. It should be noted that a number of bacteria usually considered hydrogen-sulfide-negative, including *Salmonella paratyphi*, several *Aerobacter* species, *Escherichia coli*, and several *Shigella* species, do produce hydrogen sulfide as demonstrated by the use of bismuth and cobalt-nickel indicators. The bismuth and the cobalt-nickel media were both superior to the other media in respect to the total number of positive tests obtained. Where several media yielded positive tests, the cobalt-nickel medium usually gave the most intense positive test, or at least a test as strong as any others obtained. One noticeable exception occurs in the case of *Eberthella typhosa*, which causes intense blackening of the Kligler iron medium, but which produces no visible reaction in the cobalt-nickel medium.

Ferric ammonium citrate tryptone agar was also prepared, according to directions in the Difco Manual (1939), and compared with the above media. However, the results obtained with it were not satisfactory. This may be due to the fact that the sulfur source in the commercially prepared material is better sensitized

to the action of bacteria than that in the medium made and used in these investigations. (Private communication from Dr. H. G. Dunham of the Difco Laboratories.)[2]

These results indicate that certain of the media devised for the determination of hydrogen sulfide production by bacteria fail to agree among themselves. *Shigella* species and *Streptococcus lactis* give positive tests only with the bismuth indicator; *Eberthella typhosa* does not give a positive response with cobalt and nickel, but does with lead, iron, and bismuth; the strains of coliform and *Salmonella* species investigated give better tests with the cobalt-nickel medium than with any other; *Aerobacter cloacae* gives a positive test with cobalt-nickel indicator only. Some of the media compared in this work have been successfully used to distinguish various bacterial species in certain genera; this shows that the ability of an organism to produce detectable hydrogen sulfide is a function of the medium and of the indicator used to make the test. It is not enough to state merely that an organism does or does not produce hydrogen sulfide without stating the conditions under which it was investigated. Hunter (1938) was led to a similar conclusion.

Several explanations probably are involved in the lack of agreement in these experiments. One is that certain detecting ions are more sensitive to hydrogen sulfide, and hence yield positive tests while others can not. This would explain why bismuth shows hydrogen sulfide production from *Shigella* species while no other detector ion does so. In other cases some of the metal ions used may poison the enzyme system essential to hydrogen sulfide production. For example, *Eberthella typhosa* gives positive tests in lead, iron, and bismuth media, and a negative test in the cobalt-nickel medium; possibly the latter ions inhibit some essential enzyme system of that particular organism. Similarly, the lead ion may poison the enzyme system of *Sal-*

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[2] Dehydrated cobalt-nickel media of the above composition (with the sulfur source omitted) was prepared through the kindness of the Difco Laboratories. When 0.2 per cent cysteine was added, results obtained were identical with those from the media prepared according to the above formula. The Difco experimental medium is known as #S-34010B.

monella paratyphi, while bismuth, cobalt, and nickel ions do not. Thirdly, hydrogen sulfide production undoubtedly depends upon the ability of bacteria to cleave the material serving as a sulfur source. Desnuelle and Fromageot (1939) have demonstrated the presence of an adaptive enzyme, "cysteinase," produced by *Escherichia coli*, which splits cysteine to form hydrogen sulfide. It may be that with the coliform bacteria the cobalt-nickel medium gives better tests than do the other media for hydrogen sulfide because only the former medium contains added cysteine as a sulfur source. This agrees with the findings of Levine (1936). Inhibition of growth by the metal detector ions is not an acceptable explanation, as every species grew in all of the media investigated.

It is interesting to note the wide difference in toxicity displayed toward bacteria by salts of the three metals, iron, cobalt and nickel, considering that the three elements are so similar chemically. Cobalt, although immediately between iron and nickel in the periodic system, is appreciably more toxic to bacteria than are either of the latter metals. The general maximum tolerated concentrations of iron (ferric), cobalt, and nickel ions are in the approximate ratio 50–1–5, respectively. Since these three elements are in the same period and column in the periodic table, and have very similar physical and chemical properties, this difference in toxicity is of interest.

An incidental observation in this investigation was that the presence of added cysteine in the cobalt-nickel medium prevented the usual fermentation of glucose with the formation of gas, which is characteristic of the coliform and other enteric bacteria. This is possibly due to the fact that' the free glucose in the medium was used up in chemical combination with the excess of cysteine present (Schubert, 1939), and, thus, was not subject to normal attack by bacteria.

SUMMARY

1. The toxicities of nickel and cobalt ions for a number of bacterial species in broth cultures were determined.

2. A medium containing proteose peptone, cysteine, glucose,

dipotassium phosphate, nickel and cobalt nitrates, agar, and water was developed which compares well with present established media for detection of hydrogen sulfide production by bacteria.

3. Organisms usually considered to be negative hydrogen-sulfide producers, such as *Salmonella paratyphi*, *Streptococcus lactis*, *Escherichia coli*, and a number of *Aerobacter* and *Shigella* species, give positive reactions with bismuth or cobalt-nickel indicators, or both.

4. It has been shown that detection (and perhaps production) of hydrogen sulfide in bacterial cultures varies greatly according to the medium and indicator employed. A statement of the ability of a microorganism to produce hydrogen sulfide is of little value unless all factors are specified.

5. The possible explanation of this variation in the detection of hydrogen sulfide production by bacteria has been discussed.

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PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

CENTRAL PENNSYLVANIA BRANCH

THE UTILIZATION OF HYDROCARBONS BY BACTERIA. *R. J. Strawinski and R. W. Stone.* The Pennsylvania State College.

Mixed cultures of microorganisms, capable of utilizing pure hydrocarbons, were obtained from the soil by the use of an enrichment medium containing mineral salts and hydrocarbon as the sole source of carbon. Nitrogen in the form of $(NH_4)_2SO_4$, a temperature of 28°C. and a plentiful supply of air were favorable for the utilization of the hydrocarbons.

The microorganisms are motile gram-negative rods which form white, iridescent, or yellowish-green colonies on nutrient agar. Plate counts were made on nutrient agar, pH 7, after eleven consecutive transfers in the hydrocarbon medium. The counts were interpreted as indicative of the relative utilization of the hydrocarbons by bacteria.

Cetane, naphthalene, and biphenyl were the most rapidly attacked of eighteen compounds studied, with counts ranging from one to one and one-half billion microorganisms per ml. of solution. N-octane, alpha-methyl naphthalene, tetralin and tertiary butyl benzene were also readily attacked. Iso-octane, 1- and 2-octenes, decalin, n-butyl benzene and iso-butyl benzene were utilized to some extent, showing counts of 30–100 million bacteria per ml. Attempts to develop cultures capable of growing on benzene and toluene were unsuccessful.

SOME BACTERIA OF THE ANTARCTIC. *Chester A. Darling,* Allegheny College.

On the second Byrd Antarctic Expedition, A. Siple collected material which was sent to Allegheny College for bacteriological study. This material consisted of snow samples, debris of mosses, algae, and lichens and soil, and included petri dishes exposed and inoculated in Little America. These collections were made in three general regions; at Queen Maud Mountains on the edge of the Antarctic plateau, in Marie Byrd Land, and in the vicinity of Little America.

One hundred-seventy-nine cultures have been isolated and studied; all but five have been classified. One hundred seventeen have been found to be members of *Bacillus*, representing the following species: *B. mesentericus, subtilis, tumescens, malabarensis, cereus, fusiformis, megatherium, consolidus, albolactis,* and *sylvaticus.* Strains of *B. mesentericus* comprised 44 per cent of the isolations.

Forty-five cultures of non-spore-forming rods included *Achromobacter liquidum, A. delicatulum, Flavobacterium devorans, F. flavescens, F.*

solare, F. sulphureum, F. turcosum, Proactinomyces agrestis, and *Serratia marcescens.*

Seventeen cultures of cocci included *Micrococcus caseolyticus, M. flavus, M. freudenreichii, M. halopilus,* and *Gaffkya tetragena.* Three yeasts and several molds were also isolated.

REGIONAL ILEITIS. *Arthur Brumbaugh*, Altoona, Pa.

Eight years ago, Crohn called attention to a disease which he named regional ileitis. Several hundred cases have been reported in the past eight years, almost fifty per cent of which had a previous history of appendicitis. Almost all cases reported have had a history of some previous abdominal trouble.

One of the sequelae most dreaded by surgeons after a laparotomy is mesenteric thrombo-phlebitis. It not infrequently produces complete blockage of the venous return from a portion of the intestine, with gangrene of the part involved and death of the patient.

A study of regional ileitis suggests that this condition is preceded by less severe types of thrombo-phlebitis of the mesenteric veins which produce a chronic passive congestion and hypertrophy of the corresponding mesentery and intestine. This is analogous to thrombosis of the iliac vein following puerperal sepsis which sometimes results in a permanent enlargement of the corresponding lower limb.

OBSERVATIONS ON THE MICROFLORA OF AGEING CIGARETTE LEAF TOBACCO. *W. E. Grundy* and *J. J. Reid*, The Pennsylvania State College.

A study has been made of the morphological and cultural characteristics of the bacteria found upon ageing flue-cured tobacco. The isolations studied were obtained from 185 samples and included more than 3500 cultures.

Pigmented gram-negative rods were found to constitute the greater part of the flora in the early stages of the ageing process. Members of *Flavobacterium* proved most numerous.

The physiological activity of the flora appeared to be directly related to the composition and quality of the leaf. Samples of low sugar content and inferior quality were found to be characterized by a flora of yellow-pigmented, monotrichous rods which do not attack gelatin and produce only slight acidity in glucose-peptone broth. Tobacco of somewhat better quality was found to be characterized by yellow-pigmented peritrichous rods which attack a number of the common sugars with the production of acid and liquefy gelatin. Flue-cured tobacco of the highest quality was found to be characterized by the presence of yellow-pigmented peritrichous rods which form acid and gas in the presence of most of the common sugars and attack gelatin vigorously.

KENTUCKY BRANCH

FIRST MEETING, JOSEPH E. SEAGRAM AND SONS, INC. DISTILLERY, LOUISVILLE, KENTUCKY, JUNE 28, 1940

STUDIES ON ALCOHOL TOLERANCE OF YEASTS. *William D. Gray*, Research Department, Microbiology Division, Jos. E. Seagram & Sons, Inc., Louisville, Ky.

Twenty-two species and strains of yeasts, largely of the genus *Saccharomyces,* but also including representatives of the genera *Schizosaccharomyces, Torula, Zygosaccharomyces,* and

Willia have been investigated in order to determine their abilities to utilize sugar under conditions of various alcohol concentrations.

Results based on sugar analyses of twenty-four hour fermentations show that, in general, the per cent of sugar utilization varies inversely with alcohol concentration. Great differences occur between various yeasts in their abilities to tolerate alcohol; hence, for any given alcoholic fermentation some care should be exercised in the selection of a proper yeast strain.

Experiments conducted with the Warburg-Barcroft manometer show that alcohol concentration strongly influences fermentation rate. Rate of fermentation bears the same relation to alcohol concentration as does percentage sugar utilization; i.e., the lower the alcohol content of the medium, the faster the rate of fermentation.

THE INCIDENCE OF SALMONELLA IN NORMAL HOGS. *Harvey L. Rubin,* Department of Bacteriology, University of Kentucky, Lexington, Ky.

The mesenteric lymph glands from apparently normal hogs were examined for *Salmonella.* Of 40 lots of hogs, consisting of 25 animals each, 19 or 47.5 per cent of the lots yielded *Salmonella* organisms. Two hundred and twenty-four cultures of *Salmonella* were obtained and one hundred of these have thus far been examined. The following *Salmonella* types have been obtained: *S. choleraesuis* var. *Kunzendorf, Salmonella sp.* (Derby type), *S. anatis, S. enteritidis, S. typhimurium, Salmonella sp.* (Bredeney type), (Muenchen type), (St. Paul type), (New Brunswick type), and a hitherto undescribed type which has been named "*Salmonella Lexington.*" The new type has the following antigenic formula: III, X, XXVI: z_{10}: 1,5. . . . Several

of the large lots and one of the single lots, so far examined, have contained more than one type of *Salmonella.*

OBSERVATIONS ON THE DISTRIBUTION OF PSEUDOMONAS FRAGI. *H. B. Morrison and B. W. Hammer,* Kentucky and Iowa Agricultural Experiment Stations.

The frequency with which *Pseudomonas fragi* causes defects in dairy products makes its distribution of importance. Previous investigations have shown that the organism is often present in raw milk and cream and other dairy products from Iowa and surrounding states. In this study it was isolated from 29 of 176 samples of milk delivered during the cool seasons to an Iowa plant. It was also demonstrated in 16 of 40 samples of milk delivered to a Kentucky milk plant in December but was not found in 17 samples delivered to the same plant in June.

Samples of soil, water, feed and bedding and swab cultures from milking utensils, miscellaneous barn equipment, floors, ledges and the cows themselves were investigated. Seventy-one of 137 such samples obtained on Iowa farms during the winter and spring yielded *P. fragi* and 37 of 99 samples obtained on Kentucky farms in the winter yielded it, but it was found in only 2 of 49 samples obtained on Kentucky farms in the summer. Studies on barnyard soil from many of the states indicate that *P. fragi* is widely distributed geographically.

EFFECTS OF SOFT X-RAY RADIATIONS ON SERRATIA MARCESCENS AND PSEUDOMONAS AERUGINOSA. *Elizabeth V. Wright,* University of Kentucky and Harold J. Kersten, University of Cincinnati.

Cultures of *Serratia marcescens* and

Pseudomonas aeruginosa were exposed to the radiations of a copper target gas x-ray tube operated at 28 kv. and 10 ma. for ½ minute periods through 160 successive irradiations and subcultures on 3 per cent glycerol agar. Heavy capsules appeared on the cells of both species as a result of these irradiations. Pigment production was not affected in either species. Cultures from irradiated *Pseudomonas* cultures also developed clear plaque-like areas containing heavily encapsulated organisms.

In a second series of studies agar plates were streaked with *Serratia marcescens*, one-half of each plate covered with sheet lead to serve as non-irradiated controls and irradiation and incubation carried out simultaneously for 64 hours. As a result of this treatment discrete colonies became fluid. Broth washings of these fluid colonies did not yield a transmissible lytic agent. Berkefeld filtrates of the washings contained filterable forms which grew out to produce red pigmented broth cultures when left at room temperature for a few days.

A SPORE-FORMING LACTOSE-FERMENTING ORGANISM FROM THE LEXINGTON WATER SUPPLY. *David B. McFadden*, Department of Bacteriology, University of Kentucky, Lexington, Ky.

A spore-forming, lactose-fermenting organism has been isolated from the Lexington, Kentucky, water supply. This organism is considered to be responsible for the frequent positive and doubtful presumptive tests which are obtained from this water supply. This organism, when first isolated, was apparently microaerophilic since it grew only as subsurface colonies on poured agar plates. It later adapted itself sufficiently to grow moderately well under aerobic conditions. It most closely resembles *Clostridium tertium* and *Bacillus aerosporus*. It differs from *Clostridium tertium* in that it liquefies gelatin, ferments inulin, and does not have strictly terminal spores. It differs from *Bacillus aerosporus* in its failure to grow on Endo or eosin methylene blue agar plates, and to digest or "black rot" carrots. Also it does not produce central spores.

CONNECTICUT VALLEY BRANCH

SPRING MEETING, MASSACHUSETTS STATE COLLEGE, AMHERST, MASS.,
MAY 18, 1940

GRANULE-LIKE BODIES IN A STRAIN OF STREPTOCOCCUS. *Ralph L. France and Gertrude Hadro*, Department of Bacteriology, Massachusetts State College.

In a strain of streptococcus, probably an atypical *Streptococcus salivarius*, granule-like bodies were observed inside the cells after 10–15 hours cultivation in a 0.5 per cent glucose broth. It was found necessary to use rather vigorous staining methods to reveal these structures. Microchemical tests for volutin were negative. Conclusive evidence of budding has not yet been observed. The possibility of spore formation was eliminated as well as the possibility of autolysis. This condition was not observed in the cells when the pH of the broth was kept above 6.0. It is the opinion of the authors that these granule-like bodies appear in certain phases of the growth cycle of the organism.

OBSERVATIONS ON THE PATHOGENICITY OF AVIAN STAPHYLOCOCCI. *W. N. Plastridge and Erwin Jungherr*, De-

partment of Animal Diseases, Storrs Agricultural Experiment Station, Storrs, Connecticut.

The biochemic and pathogenic properties of avian staphylococci obtained from joint, skin and navel-ill lesions of barnyard fowl were determined.

A group of strains derived from joint and skin lesions were lethal to six-weeks-old chickens, on intravenous injection, and caused severe intradermal reactions in chickens (wattle) and rabbits. The different members of the group were similar biochemically; they produced alpha toxin and coagulated rabbit plasma. They differed from staphylococci that are pathogenic for man by peptonizing litmus milk, as a rule, by slow, instead of rapid, fermentation of lactose and mannitol, and by growing on alkaline bromthymol blue lactose agar without causing a change in the color of the medium.

Non-lethal strains were derived from natural and induced fowl pox lesions, a comb-grown vaccine, and lesions which also yielded lethal strains. All failed to produce alpha toxin and to coagulate rabbit plasma. Several produced mild skin reactions.

Navel-ill strains were comparatively inactive biochemically; they failed to produce hemolysin, to coagulate rabbit plasma and to produce distinct reactions in chickens. Very slight intradermal reactions were produced in rabbits.

The ability of avian staphylococci to coagulate rabbit plasma is a reliable means of detecting definitely pathogenic cultures of avian staphylococci.

AN UNUSUAL SARCINA FORM. *H. Gilbert Crecelius*, Department of Bacteriology, Yale University.

Smit in 1933 undertook a comprehensive study of anaerobic sarcinae, and concluded that the organism he isolated from the stomach was identical with *Sarcina ventriculi* as reported by Goodsir.

The organism here described was found to inhabit the intestine of the guinea pig; it was isolated by making rapid serial transfers in tomato broth, pH 4.0, under anaerobic conditions. In this medium growth takes place within six hours at 37°C., as a flocculent sediment on the bottom of the tube. During the early hours of the extremely active fermentation the sediment is churned up by the large amount of gas evolved.

This sarcina form grows readily in poured tomato extract agar plates; but only under strict anaerobiosis and with added CO_2 can surface growth be obtained. The optimum temperature is 37°C.; feeble growth occurs at room temperature; the organism is killed at 60°C. within 10 minutes. Bacto peptone-"Savita" extract medium is an adequate source of nitrogen. To obtain growth a fermentable carbohydrate is essential, usually in a concentration of two per cent. Glucose, lactose and sucrose are attacked, whereas raffinose, xylose and mannose are not. A strong odor of butyric acid accompanies fermentation.

The packets usually contain 32 cells, the cell size averaging 3.3 to 4.0 micra. At six hours the cells stain strongly gram-positive, but after twelve hours incubation they are mostly gram-negative.

This report is presented here because the organism studied is one of unusual interest, in that as a sarcina it is strictly anaerobic, powerfully fermentative, and appears to be a part of the normal intestinal flora of guinea pigs.

A STUDY OF PROTEUS HYDROPHILUS. *David Borden and Walter L. Kulp*, Department of Bacteriology, University of Connecticut.

THE EFFECT OF SURFACE-TENSION DEPRESSANTS ON CERTAIN IMMUNOLOGICAL SYSTEMS. *Lida F. Holmes*, Department of Bacteriology, Yale University.

Three classes of surface-active agents, alcohols, soaps, and esters were introduced into serological systems in an attempt to ascertain whether such agents could modify specific aggregation by lowering the interfacial tension between the antigen particles and the menstruum.

The soaps and three of the esters employed did inhibit immunological reactions. Another ester, cetyl pyridinium bromide, exhibited an opposite behavior, that is, it decreased the stability of antigens and caused non-specific precipitation of serum. The alcohols did not alter the serological reactions in any detectable manner.

Detailed studies indicated that sodium lauryl sulphate, chosen as an example of the compounds which inhibit serological reactions, acted by its property of increasing the negative charge on the antigen, and not by denaturing the antiserum or antigen. Cataphoresis determinations with cetyl pyridinium bromide, on the other hand, demonstrated that this compound decreases the charge on the antigen surface.

It was observed that the compounds which increase antigen stability have the common characteristic of ionizing with their large, non-polar, organic portion as an anion. Cetyl pyridinium bromide, in contrast, ionizes with its organic, hydrophobic portion as a cation. It was concluded that the surface-active agents studied altered antigen stability because they formed highly-adsorbed ions, and that the alcohols were inactive because they do not ionize. The behavior of surface-tension depressants in immunological systems could be correlated with their influence on charge of the antigen particle and could not be correlated with their probable action on interfacial tension.

A STUDY OF THE DISINFECTANT CONCENTRATION EXPONENT. *Philip B. Cowles*, Department of Bacteriology, Yale University.

Evidence is offered to support the hypothesis that in the disinfectant equation $C^n t = k$ the value of n with surface active germicides is related to the surface tensions of the aqueous solutions of the substances. This same general equation holds for the wetting of wool, and the concentration exponent has the same value for both wetting wool and killing *Staphlococcus aureus*. In the case of phenol the log t/log c curves for wetting and killing and the γ log c curve all have sharp and comparable breaks at a phenol concentration of 0.08 m. In greater concentrations than this the n value for killing is the commonly accepted one of 5.5; in lesser concentrations it is 2.5. With the fatty acids, n for wetting is about 3.5, but for killing it varies from 2. for acetic to 10. for caprylic. It is argued that n for these acids is made up of two factors, that related to the surface activity of the acids and that due to the effective H-ion concentration. With H-ions furnished by HCl, n increases rapidly between pH 2.5 and pH 4, the range given by lethal concentration of the fatty acids.

STERILIZATION OF DRINKING GLASSES BY ULTRA-VIOLET LIGHT. *K. N. Atkins*, Department of Bacteriology, Dartmouth Medical School.

COLIFORM "MUTANTS," WITH RESPECT TO THE UTILIZATION OF CITRATE

LELAND W. PARR AND WILLIAM F. SIMPSON

From the Department of Bacteriology, Hygiene, and Preventive Medicine, School of Medicine, The George Washington University, Washington, D. C.

As has been shown (Parr, 1939), a test for the utilization of citrate as a sole carbon source is one of the three most widely used technical procedures for determining the identity and relationships of coliform bacteria. Most workers who have made use of it since its introduction by Koser (1923) consider the test satisfactory, whether they employ it as originally proposed (Koser, 1924a) or make use of the refinement of Simmons (1926). Since the citrate utilization test introduced new concepts into the bacteriology of coliform organisms, Koser (1924b) undertook experiments to determine if citrate utilization possessed those characteristics of stability essential to a diagnostic procedure. He showed that citrate utilization, or the lack of it, is "constant and reliable" and not readily acquired or lost. With these conclusions we are in complete accord. *Escherichia coli* does not satisfactorily utilize citrate as a sole source of carbon for growth purposes, and when sown in citrate broth (Koser test) or streaked on citrated agar (Simmons' test) fails to develop to the threshold of recognition. On the other hand, *Escherichia freundii, Aerobacter aerogenes,* and *Aerobacter cloacae* develop promptly, usually producing easily recognized growth within 24 hours.

Ruchhoft and co-workers (1931) have clearly shown that the colon bacillus does grow in Koser's citrate broth but that it only attains therein a population density of from one to five million cells per milliliter which is not enough to give a definitely recognizable turbidity. It is significant that these workers recovered typical *Escherichia coli* from such "negative" tubes weeks after

inoculation, in one case even after a year's time. They definitely state that such cultures did not exhibit any change in their biochemical features, although rough colonies were frequently encountered in platings from such aged broth sources.

Evidence exists that occasional coliform strains may be encountered which are neither absolutely negative nor completely positive with respect to the utilization in culture of citrate as a sole source of carbon. Parr (1938) reported as "mutants" certain coliform strains departing from the generally accepted rule. It remains to describe how these atypical strains behave, how "mutants" are handled to obtain pure breeding lines of descent, to give a preliminary statement as to the stability of derived lines, and to discuss the possible significance of such findings.

The senior author first observed the citrate "mutants" as early as 1934 (see Plate 1), and in his laboratory Evans (1935) induced their production by special cultural procedures. The clearest picture of what happens in nature with respect to citrate "mutants" can, however, best be obtained through a description of a culture, called H.23, which, with 64 other atypical coliform cultures, was sent to Parr by A. V. Hardy of the DeLamar Institute of Columbia University from his studies on *Shigella* from Indians carried out in New Mexico in the summer of 1937.

H.23, a slow lactose fermenter, when tested was indole positive, methyl red positive, and Voges-Proskauer negative. When inoculated[1] on a sector of Simmons' citrated agar it produced no immediate growth. H.23 was, therefore, apparently a slow lactose-fermenting *Escherichia coli* (Imvic $+ + - -$). It was found, however, that on the fifth day after citrated agar had been inoculated, one colony appeared on the area sown. This "mutant" colony grew larger and by the seventh or eighth day was accompanied by a change in color of the surrounding medium from green to blue. No other colony developed on the area despite the holding of the plate for a total of fifteen days' observation.

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[1] The citrated agar is inoculated by wiping a shaken loop of culture in broth (no visible film) on a sector of the plate the size of half a dollar.

The first impression from such results is that there has been a contamination of the medium. Subsequent work, involving the

Plate 1
Above-Rt.citrate
    positive,
    1ft.two
    "mutants."
Left--"Mutant" of
    T.18.
Below-Original
    H.23
    "mutant"
    smear on
    left.

handling of many "mutant" strains and their derivatives, has eliminated this point as a proper explanation. "Mutants" appear

regularly and constantly on citrated agar from strains known to produce them; such colonies do not appear on citrated agar when strains are under study which do not produce them; the contamination rate for citrated agar is very low and is mostly confined to molds; and, most satisfactorily, the colonies which appear as "mutants" can be shown to have biochemical and serological identity with the culture under study from which they arise.

Nor was the appearance of these "mutant" colonies on citrated agar on an otherwise negative smear the manifestation of a culture mixture. We have, for instance, carried another strain, L.W.P.6-2, from its original isolation from fresh feces through 66 "platings out" and in testing each subculture have found that colonies transferred to citrated agar gave "mutants."

Neither do we believe that this phenomenon is one common to all *Escherichia coli*, merely missed in the past because tubes and plates in most laboratories have been discarded too soon for the "mutants" to be seen. We have tested numerous strains of *Escherichia coli* on citrated agar, holding the plates for as long as four weeks for observation (one day at 37°C., thereafter at room temperature), and we do not find that this "mutation" appears on any but a very small fraction of the total. It should be noted that in this work Koser's citrate broth cannot be used. A solid plating medium is necessary to fix and isolate the colony which may develop from a cell which manifests the ability to metabolize citrate. Difco products have been used throughout.

Work with the H.23 culture proceeded along two lines, each accomplishing an end which complemented and completed the findings obtained by the other procedure.

On the one hand the tryptone broth culture of H.23, from which the citrated agar had been stroked (see above), was plated on Endo's agar October 27, 1927. Twenty of the colonies which appeared on the plates were tested on citrated agar. The technic used was to touch one of the colonies with a straight needle. A tiny bit of the inoculum so obtained was placed on a sector of a Simmons' citrated agar plate and the rest was inoculated into a tube of tryptone broth numbered to correspond to the sector on citrated agar inoculated. Then, with a loop needle, the

trace of colony placed on the citrated agar was carefully spread out over an area about the size of a quarter. The size of the spread has depended upon whether three, five or six colonies are to be tested on a single plate of citrated agar. The inoculated citrated agar plates and tryptone broth tubes have been incubated over-night at 37°C. and thereafter held at room temperature.

In the case of the 20 colonies so inoculated, deriving from an Endo's agar plate made from the H.23 broth culture, no growth was evident on the citrated agar for three days. On the fourth day a single "mutant" colony appeared on each of three of the twenty test areas. In five days a fourth sector showed one colony and in seven days a fifth sector revealed one colony. By ten days 12 of the 20 citrated agar sectors showed one or more colonies (1, 1, 1, 2, 2, 2, 4, 6, 6, 8, 9, 14). From one of the twenty broth tubes, that corresponding to a sector showing six "mutant" colonies, a plating on Endo's agar was made and 18 of the colonies developing were picked to citrated agar and to broth. This time no one of the 18 smears showed a "mutant" colony until the sixth day, when on one sector one such colony appeared. By eleven days eight of the 18 smears showed "mutant" colonies (1, 1, 1, 1, 2, 2, 2, 2).

We were able to continue this process indefinitely, always being able to show among the colonies sampled in any given subculture on Endo's agar some which when smeared on citrated agar gave rise to "mutant" colonies.

Not all of the smears made gave rise to "mutant" colonies (12 of 20; 8 of 18; 14 of 20; 5 of 20; 4 of 20; 8 of 10, and so on). In other words, the number of cells capable of "mutating" in such a culture as H.23 is not large. One may stroke out thousands of cells on an area of citrated agar without obtaining a "mutant" colony, even though there are capacities for "mutation" in the culture under test. Early in the work we satisfied ourselves that whenever, as in the work just described, a smear on citrated agar showed no "mutant," it was nevertheless possible to obtain "mutants" from the colony in question on Endo's agar. This was done by plating the broth culture corresponding to the

negative citrated agar sector on Endo's agar and testing colonies so obtained. We never failed to obtain in any such check one or more "mutants" to demonstrate that the capacity for "mutation" was not absent.

H.23 was continued in this way for 15 sub-cultures in the preliminary work, during which 188 colonies on Endo's agar were tested, and there was no break in the constant yield of "mutant" colonies in each sub-culture. In no case was a typically positive, prompt citrate reaction obtained. As noted above, certain smeared sectors failed to reveal "mutant" colonies, but in each case it was possible to show that the Endo's agar colony smeared contained cells which could "mutate." Since it was found that few if any "mutant" colonies developed on a citrated agar sector after ten days, this time interval was adopted as the minimum time for holding the citrated agar plates. We have observed the appearance of "mutants" in much the same way in each of the seven other coliform cultures studied in detail,—T.16, T.18, T.20, P.C.14, L.W.P.6-2, and L.W.P.11-3. Were no other data available we should be inclined to think of the phenomenon as merely another instance of the "unstable variant" described by Deskowitz, (1937). See diagram 1.

However, when we gave attention to the "mutant" colonies which appeared on the citrated agar, the findings could not be so readily explained. Thus, to illustrate our second approach to the problem, the "mutant" colony which appeared, October, 1937, on a sector of citrated agar was touched lightly with a needle and inoculated into broth which, after incubation, was plated on lactose indicator agar. Twenty of the resulting colonies were fished, as above, to sectors of citrated agar and to broth. This time, however, 19 of the 20 sectors showed on over-night incubation the typically positive citrate reaction, i.c., growth uniformly over the entire area smeared, and a color change in the medium from green to blue. One sector of the 20 was negative and remained so during the fourteen days of observation. On it no "mutant" colony appeared at any time.

The broth corresponding to this negative sector (C.162) was plated on Endo's agar and 20 colonies were picked to citrated

agar and broth.   All 20 sectors were entirely negative during a period of observation of 14 days.   Again, a broth culture from

Tryptone
broth culture
of H.23

Endo's Agar

Broth

Citrated Agar
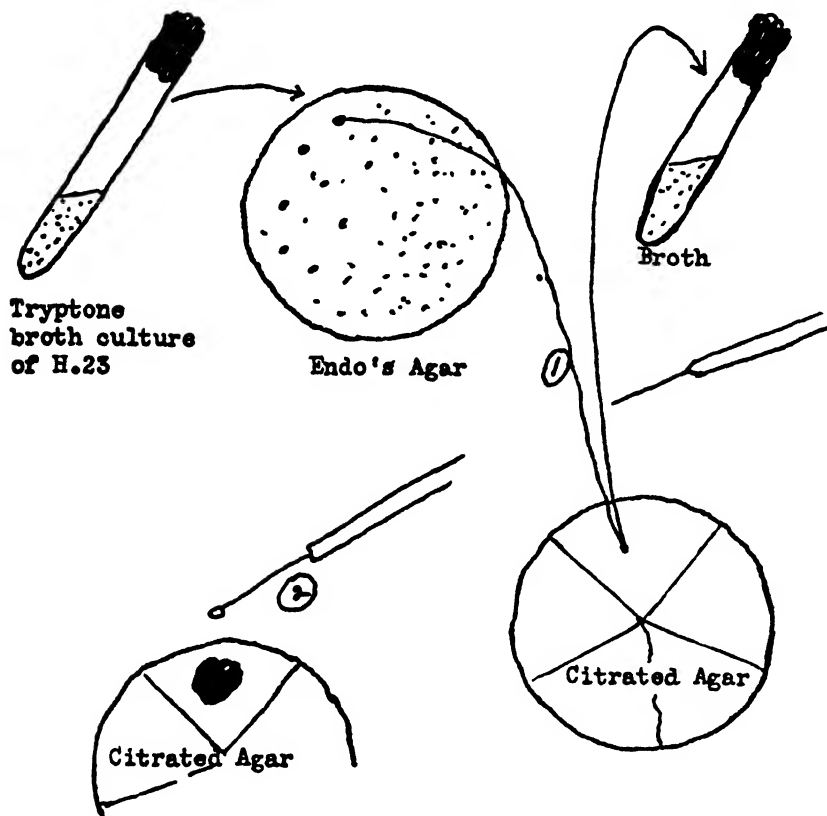
Citrated Agar

This perpetuates the "mutant" in series.

Broth →Endo →Broth →Endo →Broth → et seq.
         ↘Citrate   ↘Citrate

DIAGRAM 1

this series (C.245) was plated and another lot of 20 colonies picked to citrated agar and broth.   As before no growth of any kind was observed on the citrated agar sectors inoculated.   This

procedure was repeated until 19 subcultures involving a test of 194 colonies from Endo's agar had been made. No one of the 194 grew on citrated agar or gave rise thereon to "mutant" colonies.

On the other hand, when a broth culture corresponding to one of the 19 positive sectors was plated (C.163) and 20 colonies fished to citrated agar and broth, growth occurred on all 20 sectors promptly, completely, and typically. From this sub-culture, broth C.265 was plated on Endo's agar and 20 colonies picked. Again on citrated agar all sectors streaked were positive. This procedure was repeated until 22 sub-cultures, involving 187 colonies, had been tested without a single negative appearing among the 187 sectors inoculated on citrated agar (see diagram 2).

Thus, from one single parent strain, H.23, we were able to develop three true-breeding lines differing with respect to their utilization of citrate as a sole carbon source. There was, first, the citrate-unstable form which when streaked on citrated agar shows no visible growth for several days, after which growth occurs, but only as one or more isolated, "mutant" colonies, and not as a uniform film of growth covering the entire area inoculated. If sub-cultures on Endo's agar are made from the broth corresponding to such a streaking on citrated agar, colonies picked from the plate will reproduce the picture. If, on the other hand, further work is carried out from one of the "mutant" colonies instead of from the broth from which is was derived, one may obtain a positive citrate-utilizing line which develops quite as promptly and completely as would a culture of *Escherichia freundii*. And, thirdly, also starting with a "mutant" colony, one may obtain a line which refuses citrate completely, behaving exactly as would *Escherichia coli*.

This is the typical picture which we have obtained for all strains we have studied in detail, eight in all, with one exception. P.C.14 has not as yet yielded all three of the lines, but may do so with further manipulation. It is obvious from a consideration of the factors involved that one may not on first trial obtain these three lines, and that our success in doing so was in part good luck. We have almost always found that when we studied a

"mutant" colony yielding pure positives and complete negatives, the positives greatly outnumber the negatives. Chance selection of twenty colonies from an Endo's agar plate may well fail to include a negative. To date we have made detailed study of 107 colonies appearing as "mutants" on otherwise negative citrated agar sectors. Forty-eight of these when put into broth and plated on lactose indicator agar yielded, as far as tested, only positive progeny. (Obviously all colonies appearing on a plate could not be tested.)

Again it must be remembered that citrated agar is not toxic to citrate-negative organisms of the coliform group. Such forms probably persist, even multiply below the recognition threshold for some time. A "mutant" colony lies over, even contains, some of these negative cells. Furthermore, it is not improbable that when the "mutant" colony starts to develop, its metabolism of the citrate molecule presents the immediate environment with food materials which citrate-negative coliform cells can use. Thus, when such a "mutant" colony is propagated in broth and plated out, it may yield progeny negative to citrate, and some which "mutate" on citrate, as well as the derived positive forms. Thus, among the 107 "mutant" colonies studied there have been some like "Colony on C.6734," which was planted in broth and after incubation plated on Endo's agar. When 30 colonies were picked from the plates there were 19 positive sectors on citrated agar, three negative smears, and eight areas on which "mutants" appeared. If, however, the worker is careful to touch a "mutant" colony at its highest point of elevation, and deals with such colonies as soon as they can be handled safely instead of waiting until they have remained on the plate a week or longer, he is likely to obtain progeny which are either mostly positive with a few negatives, or entirely positive.

If this phenomenon is merely another instance of the "unstable variant," so well described by Deskowitz (1937), it is hard to understand how absolutely negative lines may arise, as they undoubtedly do. According to the theory of the unstable variant, each single cell possesses the capacity to develop the variant. *Bacterium coli-mutabile* is probably an "unstable variant."

Every cell in such a culture possesses the potentiality for fermentation of lactose when cultured on a substrate containing that sugar.   But lines entirely negative to lactose are not encountered. In the work here reported we have had not the slightest difficulty in isolating negative lines which breed true and persist as negative through many sub-cultures.   One may postulate that when a "mutant" colony develops, it contains many cells which have taken on the capacity for the prompt utilization of citrate but it also contains some cells which have no such capacity at all, and it may contain, from the underlying film of inoculum out of which it grows, cells which have not as yet "mutated," but which do so when the "mutant" colony is fished to citrated agar for study. The theory advanced by Reed (1933), to explain bacterial "mutation" on the basis of unequal cell division with respect to some gene-like determinant would explain such a phenomenon.

The essential identity of the three lines derived from a single parent strain has been established by comparisons of their morphology and biochemistry and by serological tests using sera prepared with the parent strains as antigens.   For H.23, 13 strains were used—the original, four other "mutating" strains, four negative strains, and four positive strains.   For the culture T.16[2] nine strains were used—the original, one other "mutating" strain, three negative strains and four positive strains.   These strains were simply well defined and stabilized cultures picked out from the complex family trees of H.23 and T.16 at wide inter-

[2] T.16 was isolated by the senior author from long-stored feces and placed in his type collection as an atypical coliform intermediate.   When first isolated and stabilized it developed promptly and typically on citrate and had the Imvic formula $++-+$.   When tested on citrate, June 30, 1937, it was at first negative, but by July 5, it had developed "mutant" colonies and when again tested on citrate December 6, 1937, it was again negative on citrated agar for several days following which one colony appeared on the otherwise negative smear.   T.16 was probably originally an *Escherichia coli* which in long stored feces (in the cold room) acquired the character of citrate utilization, but not so perfectly or completely but that years later in culture it reverted to its original reaction, *Escherichia coli*, with a potentiality for citrate mutation.   We suspect that many $++-+$ (Imvic) strains are of this type.   Such strains well illustrate Adami's (1892) concept of the development of bacterial races, an old paper which should be read by all bacteriologists.

vals in the study so as to clearly test the essential relationships and identities.

All 13 of the H.23 strains were culturally alike except for citrate utilization and the same was true for the nine strains of T.16.   With antisera prepared with the original parent strain as antigen, in each of the two cases, all 22 derived strains agglutinated either to titer, one tube below titer or one tube above titer, which seems quite satisfactory in view of the alleged serological heterogeneity of coliform bacteria.   Absorption tests were not carried out and as yet no serological tests have been done with strains other than the 22 H.23 and T.16 cultures.

The study of the citrate variants has been by no means confined to the mere derivation of the 22 strains mentioned above.   Many other cultures have been given considerable manipulation in order to arrive at some notion of their stability.   A "mutant" must not only present a new character believed to be significant, but it must exhibit the new character with reasonable constancy. For example, the nine strains of T.16 were cultivated in series through 13 passages of lithium chloride broth (6/2/38 to 6/23/38). At the end of the series the nine were tested for their action on citrated agar and seven of the nine bred true; two, both considered negative, gave "mutant" colonies (1, 2) upon prolonged cultivation on citrated agar.   Again, these nine strains were cultured in series through 17 passages of colchicine alkaloid broth (0.1 per cent in Tryptone broth) and after four weeks of constant exposure to the influence of this chemical, were sown on citrated agar.   In this series eight of the nine strains bred true, the ninth, a negative, giving one "mutant" colony on the citrated agar sector on the fifth day.   The negative derivative, T.16 no. 5, both "mutants" and all four positives, bred absolutely true to type in all of these experiments.   These facts seem to emphasize that there are differences in the degree of fixity of the characters of a new race, which is exactly what Adami postulated more than forty years ago.   At the time the preliminary work with H.23 and T.16 was done in 1937 and 1938 we were inclined to think that the stability of the derived positive was much greater than that of the derived negative, for during that period all of

our derived positive strains bred true.   More recently we have encountered some which yield a few negatives as well as many positives when cultured, but right along with them we have strains like H.23 no. 12 which has been subcultured (up to March 11, 1940) 50 times in the past year, following a period during which it was held in the ice box in the desiccated form.   In this past year's work with H.23 no. 12, 714 colonies on Endo's agar have been tested and each one of them has been completely, promptly and typically positive on citrated agar.   Study is in progress on the stability of derived citrate variants.   We already know that many of them are stable, quite within the limits of what might be expected from any normal strain of bacteria; but it must be admitted that we encounter other strains in the same family (H.23 or T.16, for example) for which the stabilization is less perfect.   If there is any mechanism for inheritance among bacteria which may eventually be made out it will have to explain imperfect stabilization of "mutants" as well as provide an understanding of how the new forms arise.

It has been indicated above that the citrate "mutant" does not occur so frequently as to endanger the value of the citrate utilization test in coliform studies.   We have studied 9924 cultures of colon-typhoid organisms including numerous strains from fresh and from stored feces, atypical coliform cultures from A. V. Hardy, strains from infectious diarrhea of the newborn, *Salmonella* types, *Eberthella* and *Shigella* types, and paracolon types.   In all, we have encountered 83 citrate "mutants" from 21 specimens or sources.   These 21 include one fresh fecal specimen from which 54 of 60 colonies studied in detail were citrate "mutants."   They also include Evans' derived coliform "mutant" which he produced in our laboratory in 1935 from culture T.24.   This organism was originally *Escherichia freundii* (Imvic − + − +) isolated more than fifteen years earlier by Koser from soil.   It was one of twelve coliform organisms with which Evans was working in a study on the effect of holding cultures of coliform bacteria for long periods of time in Stearn's gentian violet broth (1923).

Of the 12 organisms Evans had under study only T.24 was

appreciably altered.   How it lost its ability to utilize citrate is described by Evans (1935): "This change occurred gradually, there being first delayed citrate utilization, then little by little citrate-negative colonies began to predominate over citrate-positive ones, until when the experiment was suspended, 6/5/35, with one exception all colonies picked from Endo's plates produced only, for a substantial amount of inoculum rubbed on citrate plates, from one to thirty positive colonies, these colonies appearing four to five days after inoculum was rubbed on the plates.   The exception noted was that the inoculum from one colony on Endo's medium, transferred to nutrient agar and

Citrated Agar
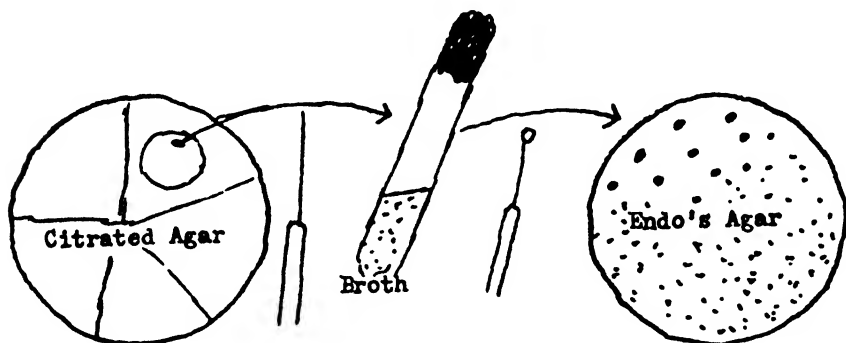
Broth

Endo's Agar

DIAGRAM 2. NOW PICK FROM THE COLONIES ON THE ENDO'S AGAR PLATE TO BROTH AND CITRATED AGAR AS OUTLINED IN DIAGRAM 1

For instance, H.23 of November 2, 1937 on Endo's 20 colonies fished, of which 19 positive on citrated agar, promptly, completely and typically.   1 negative on citrated agar, two weeks incubation, no "mutants" appearing.

repeatedly tested out on citrate never produced any citrate-positive colonies up to the time the experiment was interrupted." Evans' data are significant particularly because they relate to a much-tested strain considered as a typical *Escherichia freundii*. Significant also, as we see it, is the fact that the treatment given by Evans to all the strains with which he worked produced results in only one culture.   Exceptions like this to the general outcome of an experiment, which have generally been disregarded in the past by many workers as something to be thrown out or excused, may in our opinion be the best part of the experiment.   We are firmly convinced that bacteriologists must recognize differ-

ences in stability of bacterial strains, and where different laboratories are using different strains, or even identical strains, sometimes the same procedure may not yield the same results.

The citrate "mutant" has been of peculiar interest to us because it represents a phenomenon occurring in nature. With the exception of Evans' work, which we regret has never been extended, all "mutations" dealt with have been encountered in the regular line of cultural procedure. The forms described and the lines derived have not arisen through the use of X-rays, radium or special chemicals. Citrated agar has been used as far as possible only as an indicator, and the general propagation and plating out of strains has been done with broth and on lactose indicator agar on which all types concerned grow equally well.

Our data are not offered as an unfavorable criticism of the citrate utilization test. In fact, the results attest, in general, to the stability of coliform organisms with respect to their utilization of citrate as a sole source of carbon. We suspect that if other tests, such as the Voges-Proskauer or indole production tests were equally adaptable to "mutation" study, such experiment would reveal fully as much change of character in the strains studied.

What the results do show is that bacterial taxonomy should be conservative. In dealing with strains exhibiting slight differences in character, habitat, or action on a host, effort should be made to ensure that the characteristics utilized to define new species not only show stability combined with facility and exactness of elucidation, but also correlation with other known descriptive facts.

It could be argued that in this work new species have been evolved. Michelson and Dulaney (1936) reported the transformation of *Bacterium coli-mutabile* into *Bacterium aerogenes* and Minkewitsch, Rabinowitsch, and Joffe (1936) placed cultures of *Bacterium coli* (*communis*) in soil, free from coliform bacteria, and eventually recovered citrate-utilizing, sucrose-fermenting forms. In our laboratory Evans would seem to have changed *Escherichia freundii* into *Escherichia coli* with intermediate citrate "mutating" forms appearing along the route. As species are now recognized, our own "transformations" have been only as between *Escherichia coli* and *Escherichia freundii* of atypical

nature. We do not believe that the significance of this work lies in any transformations made *per se*, but in the demonstration that "mutations" may occur from which pure lines may be derived.

The citrate "mutant" has, moreover, provided us with an apparently new mechanism for the ready study of "mutation." In dealing with slow-fermenting forms it is easy to obtain the prompt fermenter and to preserve the slow-fermenting *mutabile* type, but the absolute negative does not appear. The citrate "mutant" yields three lines, the variant, the positive, and the negative. Further study with it may lead to a better understanding of bacterial "mutation."

One of the puzzling factors about the citrate "mutant" and about the slow-lactose-fermenting forms is the delay in appearance of the "mutant" after the inoculation of suitable material has been made. The whole theory behind the purification of a bacterial culture by plating it on a solid medium from a growth or suspension in fluid medium is that the inoculum is spread so diffusely that at many points single cells are deposited, from which distinct colonies grow. Certainly if a culture of coliform organisms which utilizes citrate be spread on a plate of citrated agar, distinct growth will be visible the next day. In the case of the citrate "mutant," however, a smear on citrated agar usually shows no sign of a colony for at least three days and frequently this time interval may be four, five, or six days. Why the delay? Secondary colonies on *Bacterium coli-mutabile* appear on lactose indicator agar only after a lapse of days. Typical cultures of this organism sown in lactose broth may show no visible acidity for more than a week. We feel that we shall never have the exact answers to what actually does go on in strains like these until methods better than those now in use have been developed.

CONCLUSIONS

A description has been presented of a coliform citrate "mutant" which can be so handled as to yield true breeding lines of the unstable variant, of a stable positive citrate-utilizer, and of a stable negative citrate-utilizer. The method used in obtaining

these three lines is described.   The probability that such forms represent contaminations or mixtures is ruled out.   It is felt that new material, valuable for the study of bacterial genetics has been presented, and that the evidence that bacterial taxonomy should be conservative has been added to.   The need for improved methods of study for this "mutant" and for others longer known to bacteriologists is emphasized.   It is suggested that differences in stability may exist in different strains of certain bacterial species, with the result that in occasional studies the results obtained will depend upon the nature of the strains utilized, making it possible for good workers in different places to obtain conflicting results.

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LETHAL AND DISSOCIATIVE EFFECTS OF X-RAYS ON BACTERIA

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Since the discovery of x-rays by Roentgen in 1895 their use in experimental biology has assumed increasing importance. As early as 1898 Rieder showed that the rays from an anti-cathode could kill *Vibrio cholerae*. Work reported since then has served to establish the lethal action of the rays on bacteria, but most of the studies have been of a qualitative nature and have dealt with the killing action only. More recently, attempts have been made to determine what particular rays are lethal. However, there are very few studies on the changes which occur in the population of x-rayed cultures.

The rays emitted from x-ray tubes are electromagnetic waves whose energy is distributed in units known as quanta. In general, all rays of wave lengths greater than 1 Å (Ångström Unit) are classified as soft rays and those of wave lengths less than 1 Å as hard rays. The latter, due to their greater energy, are able to penetrate farther into substances, while soft rays are more readily absorbed. It is important that the type of radiation, whether hard or soft, be known when applying x-rays to biologic and physical problems.

For quantitative studies of the biologic effects of x-rays, it is necessary to have some reliable means of measuring their energy or intensity. Unless an ionization chamber is available so that the radiation can be measured directly in Roentgen units, the intensity may be calculated to a fair approximation by the application of the empirical law that, for a given target in an x-ray tube, intensity is proportional to the product of the tube current and the square of the tube voltage.

The type of radiation, signifying the softness or hardness of the

x-rays, depends upon the voltage applied to the tube and upon the kind of material used as the target. For a given voltage there exists a minimum value of the wave length of the emitted x-rays. This relation may be expressed by the formula:

$$\lambda = \frac{12345}{V}$$

where λ is measured in Ångström units and V is measured in volts. Unless a filter is used, the energy of the x-ray beam is distributed among all the wave lengths from this minimum to the very longest x-rays; however, most of the energy is contained in the wave length region close to the minimum value. Superimposed upon this continuous radiation is the characteristic radiation. This is dependent on the material used as the target; the higher the atomic number of the metal used, the shorter the wave length, and hence the harder its characteristic radiation. From these considerations it is evident that x-rays become harder as the tube potential and the atomic number of the target are increased.

The majority of previous studies on lethal effects of hard and soft rays have involved the use of agar surfaces for exposing the bacteria to the rays. By the use of this method, Schepman and Flecke (1926) found that soft rays killed bacteria within a few minutes, while equivalent doses of hard rays had approximately half the killing effects. In the same year, Ponzio (1926) subjected bacteria to the secondary rays from copper, molybdenum, silver, tin, antimony, barium, and lead, and reported that the characteristic radiations from the elements of higher atomic number were more destructive to bacteria than those from elements of lower atomic numbers, thereby inferring that hard rays are more destructive than soft. The work that followed, notably that of Burger (1931) and Pugsley, Oddie and Eddy (1935) substantiated the observations on the lethal action of soft x-rays. Wyckoff (1930) tested the lethal effects of X-rays emitted from copper and tungsten tubes and found that the rays killed *Escherichia coli* and *Salmonella aertrycke* in a linear exponential fashion. The cell parts involved in death were calculated to occupy a volume less than 0.06 that of the cell, and the absorption of a single quantum of x-ray energy was sufficient to cause death.

An important consideration in the use of agar as an exposure surface is the observation by Blank and Kersten (1935) that a toxic substance is formed in agar treated with x-rays. They found that such agar could not be solidified and contained a water-soluble toxic factor which inhibited the germination of spores and the multiplication of vegetative cells. This toxic substance was not found in irradiated beef extract, water, salt, or peptone. In the light of these findings much of the previous work using an agar surface as an exposure medium should be reconsidered.

Some investigators have reported that x-rays affected the physiologic and colonial characteristics of yeast and bacteria. Schneider (1926) treated actively fermenting cultures of yeast and found that 10 per cent of their fermenting ability was lost. However, when yeasts were neither actively fermenting nor in the presence of electrolytes, they resisted the action of the rays. Rice and Guilford (1931) demonstrated that x-ray treatment gave a marked increase in dissociation from "rough" to "smooth" colonies in a rapidly growing bovine strain of *Mycobacterium tuberculosis* which showed a tendency toward dissociation of this type. Stable "R" cultures of the same species and non-acid fast organisms were unaffected. Large doses of x-rays led to lethal effects. On the other hand, Bertrand (1929) found that x-rays of 2 Å had no effect on the virulence or on the quantity, quality, or rapidity of growth of *Staphylococcus aureus* and *Microsporon audouini*.

The success which the geneticists have had in using x-rays as a means of analyzing the genetic mechanisms of multicellular forms suggested the application of x-rays to the study of the hereditary mechanisms of bacteria. The present report is concerned with the lethal effects and the dissociative changes produced by hard and soft rays.

MATERIALS AND METHODS

X-ray apparatus

A self-rectifying water-cooled copper x-ray tube operated at voltages ranging from 15 to 36 kv. and with currents ranging from 5 to 20 ma. was used for the studies on lethal effects. With these voltages, the values of the minimum wave length ranged

from 0.823 Å to 0.344 Å, respectively, as determined by the law previously stated.   The Kα radiation has a wave length of 1.54 Å, which comprises most of the intensity of the characteristic radiation.   The x-rays produced at 15 kv. may be considered as soft rays, those at 36 kv. as hard rays.

For studies on dissociation, a Coolidge molybdenum tube as well as the copper tube was used.   The molybdenum tube was operated on a rectified power supply at a voltage of 35 kv. and at currents ranging from 15 to 25 ma.   Under these conditions the minimum wave length was 0.353 Å and the wave length of the maximum characteristic radiation was 0.710 Å.   Therefore, these x-rays were decidedly hard, although, due to the presence of the continuous radiation, some soft rays were present.

The bacteria to be treated were exposed at a distance of two inches from the target of the x-ray tube.   A fluorescent screen was then placed directly behind the vials so that they could be accurately located in the x-ray beam and also so that an estimate of the degree of penetration of the x-rays through the vials could be made.

Lethal effects

The bacterial suspensions were prepared in the following manner.   A culture of *Staphylococcus aureus* was purified by single-cell isolation.   Agar slant cultures were incubated for 24 hours and the growth harvested by gentle washings with distilled water.   The cells were then thrice washed in water by centrifugation and diluted to give a concentration of approximately one-half to one billion cells per milliliter, as determined by plate counts.   The absence of nutriment in the solution used to suspend the cells reduced the chances of multiplication during treatment.

To avoid the source of error introduced by the possible formation of toxic substances, paraffin-cellulose vials were used. Cellulose extraction thimbles, 10 mm. in bore and 50 mm. in length, were used to prepare the containers.   The thimbles were infiltrated and coated with melted paraffin, tightly stoppered with corks, placed in cotton-stoppered French bottles, and then autoclaved at 15 pounds pressure for 20 minutes.   After sterilization

the vials were cooled rapidly and, when used, 1.5 ml. of cell suspension was pipetted into each. The vials were kept in the sterile bottles until the time of x-ray treatment. Several vials of each series remained untreated to serve as controls.

Immediately after treatment, the vials were agitated to distribute the cells evenly and 0.2 ml. transferred from each to nutrient broth for dissociation studies. One ml. of each of the suspensions was diluted in a tenfold series to a dilution of 1:10 million, using 9 ml. water blanks, and pour plates were prepared from each of the higher dilutions (1:100 thousand, 1:1 million and 1:10 million) for survival counts. All plate counts were prepared in duplicate and incubated for 48 hours. Those plates that contained from 30 to 300 colonies were selected for determining the number of bacteria that survived.

Dissociation effects

In the study of the influence of x-rays on the hereditary mechanisms, purified cultures of *Staphylococcus aureus*, *Staphylococcus albus*, *Serratia marcescens* and *Leuconostoc mesenteroides* were irradiated with the rays from the copper and molybdenum tubes already described. The organisms were treated either as young growing broth cultures or as thrice-washed cell suspensions.

In most cases the tests on washed cells were made in conjunction with the studies on lethal effects. The vials containing the bacteria to be treated were filled with 1.5 ml. amounts of suspension. After treatment, 1 ml. was used to determine the numbers killed and 0.2 ml. was transferred to fresh broth for the studies on dissociation. The plates used in determining the numbers killed also served as material for observations on dissociation immediately after x-ray treatment. The broth cultures of treated bacteria were incubated at their optimum temperatures and daily observations were made on the character of the growth and morphology of the cells. Agar plates were streaked and colonial observations were made after 48 hours of incubation.

In the studies on growing cells, 1.5 ml. of 18-hour broth cultures were transferred to sterile paraffin-cellulose vials and treated with radiations from the molybdenum tube. After exposure, 1 ml.

of the culture was transferred to 9 ml. of nutrient broth and incubated. Random samples were made from day to day to observe any dissociation.

To minimize contaminations preparation of cell suspensions and handling of cultures were performed in a dust-free room. Nutrient agar plates which had been exposed to the air while work was being done were incubated with the test materials and served as controls on air-borne contaminations. Similarity of air-borne contaminants to the colonies found in the treated and control cultures was taken as sufficient reason to discard the suspected plates.

LETHAL EFFECTS OF HARD AND SOFT RAYS

The results of preliminary studies demonstrated that destruction of bacteria by x-rays is a function of their wave lengths. Experiments were planned to study the part played by various wave lengths, by exposure time, and by intensity of the x-rays.

The relation between time of exposure and lethal effects

Suspensions of staphylococci were exposed to the x-rays from the copper tube operated at 36 kv. and 20 ma. for 5, 10, 20, 40, and 80 minutes, after which plate counts were made.

It was found that killing was dependent on the time of exposure. The results, when plotted on the basis of the per cent of bacteria killed, yield a sigmoid curve similar to the "death curve." When a semi-logarithmic curve is made from the logarithms of survivals, a straight line results (fig. 1). In other words, the rate of destruction of the organisms is directly dependent upon the number present at any given time.

Relative importance of wave length and intensity

Suspensions of staphylococci were irradiated with x-rays from the copper tube operated at 15, 25, and 36 kv. with a constant exposure time of 15 minutes. At each of these voltages the following tube currents were used: 2, 5, 10, and 20 ma. This gave an ample range of intensities for each type of ray (as determined by the voltage) so that the relative effects of intensity as

compared to wave length could be studied.   In order to insure a uniform treatment of all of the cells in the suspensions the vials were agitated after 8 minutes exposure.

A second series of radiations was performed, using the same apparatus with the same range of voltages and intensities, but a longer exposure time.   These suspensions were exposed for 30 minutes, the vials being agitated after 15 minutes of exposure in each case.   Plate counts were made in the usual manner.   The graphs in figure 2 and figure 3 represent these data, in which the

minutes	per cent killed
5	55.8
10	64.1
20	73.1
40	87.2
80	95.5

Log of per cent survival

Time of exposure in minutes
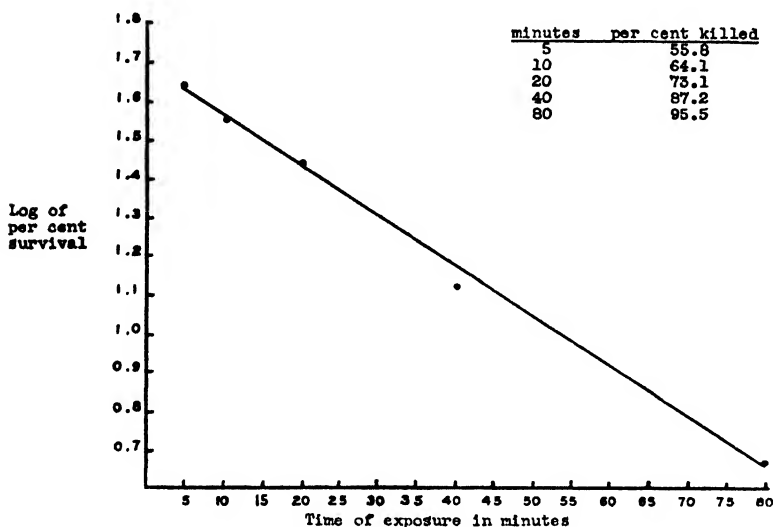
Fig. 1. Semi-logarithmic graph showing the effects of various exposure times on the survival rates of *Straphylococcus aureus*.   Copper target—36 kv. at 20 ma.

per cent of bacteria killed is plotted against the milliamperes used, with a fixed value of voltage for each curve.   The results of the second series (fig. 3) were essentially the same as those presented in figure 2, but the increase in time served to raise the total killing effects and to lessen the distance between the three curves.   The order of magnitude of the curves with respect to each other was unchanged.

The results presented in figures 2 and 3 show that the destructive power of the rays is increased as the milliamperage is raised up to a certain point.   After this point is reached, which in our

experiments, is approximately 10 ma., a further increase has much less effect on the destructive powers of the rays. For example,

kv.	ma.	per cent killed
15	5	3.3
15	10	19.5
15	20	18.8
25	2	28.0
25	5	52.5
25	10	55.3
25	20	59.1
36	2	56.9
36	5	73.2
36	10	83.5
36	20	85.3
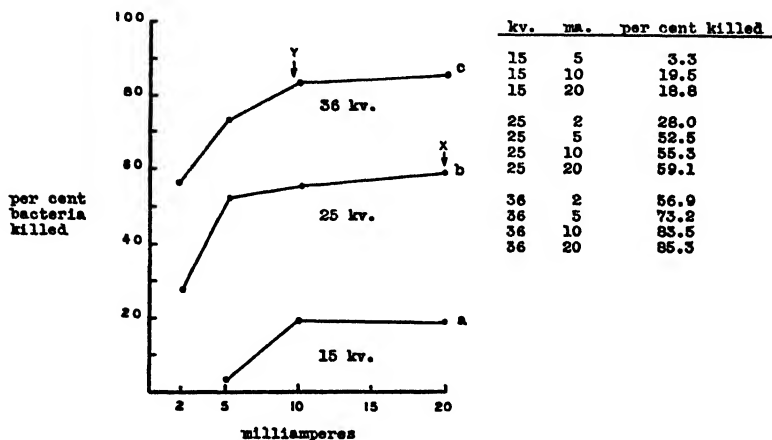
FIG. 2. Effects on *Staphylococcus aureus* of varying voltages and currents of the x-ray tube. Copper target. Exposure time 15 minutes.

kv.	ma.	per cent killed
15	5	2.8
15	10	20.4
15	20	27.8
25	2	52.9
25	5	82.4
25	10	83.5
25	20	88.0
36	2	71.2
36	5	92.6
36	10	95.4
36	20	97.2
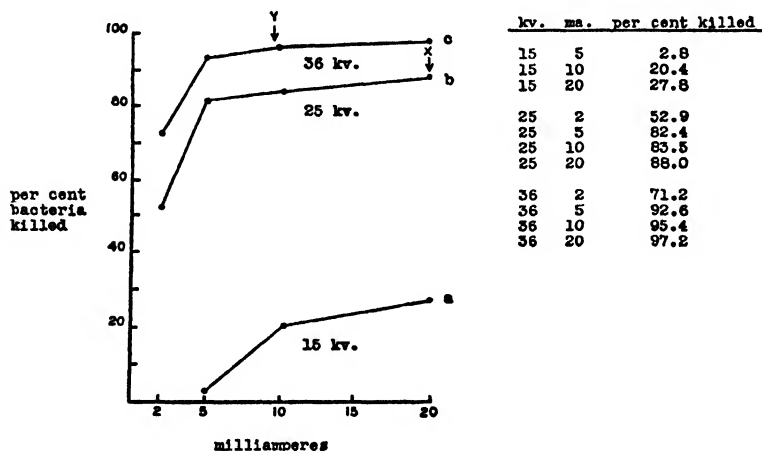
FIG. 3. Effects on *Staphylococcus aureus* of varying voltages and currents of the x-ray tube. Copper target. Exposure time 30 minutes.

at 5 ma. and 25 kv. (curve "b" of fig. 3), 82.4 per cent of the organisms were killed, while four times this milliamperage resulted

in only 5.6 per cent increase in destruction. The slope of the curve represents the per cent of bacteria killed as the milliamperage, hence the intensity, is increased. The marked decrease in the slope of the curve is explained by the fact that all of the organisms in the suspensions were not completely bathed in the rays until the intensity had become great enough to penetrate the sample completely; at this point, then, the curvature changed. The lack of penetration of the x-rays through the specimen below this point was noted by observing a fluorescent screen which was placed behind the vial.

From these curves, it appears therefore, that the type of ray is more important in the lethal effect than the intensity, and that, accordingly, hard rays are more destructive than soft rays of the same intensity. This is concluded from the following considerations.

If intensity alone were the important factor in killing bacteria, then two rays of the same intensity and capable of equal penetration should show the same lethal effects. In the relation

$$I \alpha iV^2$$
$$\therefore \frac{I_1}{I_2} = \frac{i_1V_1^2}{i_2V^2}$$

I_1 and I_2 are the intensities corresponding to the currents i_1 and i_2 and to the voltages V_1 and V_2 respectively. If these intensities are equal, then we have the condition

$$i_1V_1^2 = i_2V_2^2$$

Using this formula, one finds that 25 kv. at 20 ma. (point "x") gives an intensity equal to 36 kv. at 9.65 ma. (point "y"). However, the actual lethal effects were quite different (figs. 2 and 3). In figure 3, 25 kv. at 20 ma. gave 88 per cent lethal effects, while 36 kv. at 9.65 ma. had approximately 95 per cent. The reduction of exposure time served to make a significant difference between the points as shown by the results that for 15 minutes exposure, 25 kv. at 20 ma. killed 59 per cent of the cells in the suspension, while 36 kv. at 9.65 ma. killed approximately 82 per cent of the cells.

On the basis of the work of Muller (1927), who experimentally produced mutations in the fruit fly, it is conceivable that if bacteria have an hereditary mechanism which functions in the same manner as that of higher forms of life, this mechanism should be affected by x-rays. Alterations of this mechanism might be detected by studying the characteristics of treated cells or their progeny.

Dissociative effects on young broth cultures

Eighteen-hour broth cultures of *S. aureus*, *S. albus S. marcescens*, and *L. mesenteroides* were prepared and transferred to vials, four vials for each culture. Three vials of each culture were treated with x-rays from the Coolidge molybdenum tube operated at 35 kv. and 23 ma. using exposures of 10, 17.5, and 25 minutes. The fourth vial of each set was left untreated and served as a control. After treatment, all of the cultures were transferred to 9 ml. of nutrient broth and incubated, *S. marcescens* and *L. mesenteroides* being incubated at 25°C., and *S. aureus* and *S. albus* at 37°C. Plates were streaked daily from the sixteen cultures and observations were made on the colonies after two days of incubation.

After this period of incubation, two days after x-ray treatment, *S. marcescens* began to yield colonial variants of rough and mucoid types and color variants ranging from red to white. By the tenth day, these variations were quite striking. The control cultures yielded only a few white colonies and the remainder of the colonies were of the parent type in color and form.

The cultures exposed for 10 minutes produced several dwarfed colonies, rough types, mucoid colonies, and color variants. Those exposed for 17.5 minutes produced variants of the same types observed in the previous case, but in greater abundance. An occasional hard, pinnacled, rough, red colony was found. This type of colony seemed to have grown into the agar and had to be pried loose from the surface before isolation could be effected. The greatest number of variant types was observed in the cultures irradiated for 25 minutes, more than 50 per cent of all colonies on

streaked plates being variants.   All the forms described by Reed (1937) were present in this culture.   There were the pinnacled hard colony types, mucoid color varieties, rough mucoids, smooth white colonies, violet tinged colonies, and dwarfed colonies.

Representatives of each of the different types were isolated for the further study of their physiologic and morphologic characteristics.   Most of the mucoid forms of *Serratia* consisted of encapsulated bacilli, while those of the non-mucoid forms failed to show capsule formation on plain nutrient agar.   The isolated variants were purified by successive plating and selection.   Of the purified variants, 38 were selected for further study.

The physiologic characteristics of these *Serratia* variants were tested in glucose, sucrose, lactose, mannitol, glycerol, galactose, and salicin broths containing brom-cresol-purple as the indicator. The liquefaction of gelatin and the growth in litmus milk as well as the production of indole were also tested.   The media were inoculated and observed at 24-hour intervals.

The physiologic characteristics of the variants remained similar to that of the parent type, while the colony morphology was widely different.   However, the following alterations in physiologic properties were observed: Two of the variants failed to ferment glycerol, three formed indole, and eight produced coagulation and peptonization of milk.   The alterations in physiologic properties as compared to the parent culture did not appear to be associated with any particular changes in colony types.

After the twentieth day of incubation, all of the variants of *S. marcescens* found in the irradiated cultures were present in the controls although they were more numerous in the former.   The possibility that the results obtained were due to chance is ruled out by the fact that essentially similar results were found on repeated experiments under similar conditions.

In the irradiated broths the growths appeared red with lumpy and granular sediments.   In contrast, the shade of red of the control broth cultures was not so deep and the sediment was finely granular.   After five days of incubation the differences between the treated series and the controls were very definite.

Cultures of *L. mesenteroides* failed to show any noticeable

effects due to x-ray treatment. The staphylococci showed few changes, although some variant forms were found in the treated cultures several days before they appeared in the controls.

Effects on non-proliferating cells

The studies on lethal effects of x-rays were accompanied by dissociation studies. In the experimental series recorded in

Fig. 4 *Staphylococcus aureus* before x-ray treatment Gram stained

figure 2, it was found that the broth cultures prepared from cells treated with radiations from the copper tube operated at 15 kv. and currents of 5, 10, and 20 ma. and at 25 kv. with 5 ma. showed a distinct granular growth. This granulation was so marked that most of the growth was in this form and had settled to the bottom of the tubes as granular masses. The cellular morphology of these cultures was studied by means of the gram stain. The

control cultures showed only normal gram-positive cells (fig. 4,) while the experimental cultures containing this marked granulation were found to be composed of an abundance of streptococcus-like forms. The chains, made up of paired cocci, sometimes contained as many as 100 cells. Plates streaked from these cultures yielded only white variants and normal staphy-

FIG. 5 The streptococcus-like form occasionally appearing in *Staphylococcus aureus* cultures after x-ray treatment   Gram stained

lococci. All attempts to isolate the streptococcus-like form failed. Figure 5 shows this form as it occurred after 18 hours of incubation. After 10 days of incubation, long, gram-negative, thread-like forms appeared among the chains (fig. 6). These could not be subcultured and it was conjectured that they were formed by the fusion of cocci in a chain.

The experiment was repeated several times and in only one in-

stance were similar forms obtained. In this case it occurred in cells treated with the hard rays from copper (36 kv. and 20 ma.), while in the previous case they were found in cells treated with the soft rays from copper. The production of these forms could not be attributed, therefore, to any definite type of radiation.

Fig. 6. The streptococcus-like form of figure 5 after 10 days ageing in broth Note the tendency to chain formation. Filamentous forms can also be seen. The chain forms are gram-positive and the filaments are gram-negative.

Streaked and poured plates of x-ray-treated and washed staphylococcus cells showed greater colonial alterations with the harder rays. Variation was stimulated by soft rays, but not to the same extent as by the short wave lenghts. The examination of plates prepared immediately after irradiation showed one or two variant colonies for each several hundred parent type colonies, while the controls of untreated cells remained pure.

Effects of filtered and unfiltered rays of molybdenum

An attempt was made to determine which types of rays emitted from molybdenum were effective in stimulating dissociation in the cultures under study. A zirconium oxide filter was used to absorb the soft radiations, allowing the transmission mainly of the $K\alpha$ rays of molybdenum which have an average wave length of 0.710 Å. In this series, the time of exposure was doubled to compensate for the resulting decrease of intensity due to the absorption by the filter. In all cases, the exposures were made by the methods previously described.

Young broth cultures of *S. marcescens* and *S. aureus*, which had been previously purified by single-cell isolation, were prepared and 1 ml. of each was transferred to each of 3 paraffin-cellulose vials. One set of vials received 30 minutes exposure to unfiltered rays of molybdenum at 35 kv. and 20 ma. A second set of vials was exposed to the zirconium-oxide-filtered rays for 60 minutes, while a third set remained untreated and served as controls. After treatment the cells were transferred to nutrient broth and incubated at their optimum temperatures.

After the second day of incubation *S. aureus* showed no changes while *S. marcescens* yielded an occasional variant colony. By the ninth day there were considerable differences in the character of the sediments of the broth cultures. In the case of *S. aureus* the sediment in the control was light and readily dispersable, that of the filtered-ray-treated cultures was heavy and composed of masses, while that of the culture treated with unfiltered rays was very granular and composed of masses.

S. marcescens presented a similar picture. The control broth culture contained a red pigment with a moderately turbid growth and there was a light red ring on the side of the tube at the broth-air interface. The filtered-ray-treated culture appeared to be the same as the control, while the culture treated with unfiltered rays was deep red and contained very granular growth and a heavy red ring of bacteria on the glass at the broth-air interface. Plates streaked at this time showed that there were more colonial variations in the unfiltered-ray-treated cultures than in the filtered-ray-treated cultures. The controls yielded a few colonial variants. By the fourteenth day of incubation, the differences

between the treated and untreated cultures were very distinct, while the results of filtered and unfiltered x-ray treatments showed the same general changes both qualitatively and quantitatively.

This set of experiments supports those presented in the previous section on the production of dissociants by x-rays, and while they were not conclusive in determining what type of ray was the more effective in producing variations, they did indicate that hard rays seemed to be the more effective.

Effects of x-rays on the broth substrate

The possibility occurred to us that the x-ray treatment of broth might result in the formation of some substance that would influence the dissociation rates of bacteria, and that the results noticed were perhaps not due to the direct action of x-ray energy on the cells.   To test this possibility, several vials containing 2 ml. of uninoculated broth were treated for 30 minutes with the rays from the molybdenum tube operated at 35 kv. and 20 ma.   The irradiated broth was then transferred to 5 ml. of fresh nutrient broth and the mixture was inoculated with young broth cultures of *S. aureus* and *S. marcescens*.   For comparison, young broth cultures were treated with the same rays for 30 minutes and transferred to 5 ml. of fresh nutrient broth.   Untreated cultures were used for controls.

After 5 days, the treated cultures showed granulation and masses in the sediment of the tubes.   The controls and the culture in treated broth remained normal.   Streaked plates, made after 8 days, demonstrated that active dissociation was occurring in the x-ray-treated cultures, as would be expected from the previous experiments, while the treated broth and control cultures showed only an occasional variant.   From this it was concluded that the x-rays acted directly on the bacterial cells and not on the broth.

DISCUSSION

As is generally the case with disinfectants, both physical and chemical, destruction of bacteria by x-rays was found to occur in a semi-logarithmic fashion, indicating that the rate of destruction

of organisms was directly dependent on the numbers present at any given time. It is unlikely that the sigmoid curve resulting from plotting the percentage of survivors against the time of exposure is due to normal variability of the bacterial population in sensitivity to the rays. This implies that the individual organisms did not vary greatly in susceptibility to the rays, for if they did, a semi-logarithmic curve could not have been obtained from the results.

The cultures used in this study were purified by single cell isolation and care was taken to reduce the probability of dissociation during the preparation of cell suspensions. Studies on the dissociation rates of the parent strain of staphylococcus demonstrated that the culture purified by single-cell isolation did not show any detectable colonial variants in approximately 2,000 colonies observed on a group of streaked plates prepared by random sampling of a 24-hour-culture. This insurance of a uniform population, together with the physical aspects pointed out by Wyckoff and supported by our results, leads to the conclusion that the lethal effect is proportional to the number of bacteria present at any given time.

The results of the experiments, using tube voltages and milliamperes as variables with the time as the constant, indicate that the types of rays rather than the intensity of the rays is the important factor in killing bacteria, once the rays completely penetrate the sample.

The work of geneticists demonstrates that the treatment of plants and animals with x-rays cause mutations, but these mutations cannot be directed or predicted because of the variety of chromosomal changes produced. Muller (1927) stated that neither the detectable numbers nor types of mutations could be predicted, and found that the mutations may be of a kind already described or entirely different. He concluded that the changes produced were due to the absorption of single quanta of x-ray energy at crucial points on the chromosome.

A similar hypothesis might be adopted to explain the effects of x-rays on bacteria. One might assume that the dissociative effects are accomplished when a quantum of x-ray energy is

absorbed by an exposed cell and happens to affect a crucial point in the genetic mechanism. This effect may result in lethal effects, stable variations, or unstable variations.

No attempt was made to distinguish between lethal mutations and dead cells because of the inherent difficulties. These difficulties arise from the basis upon which cells are classified as living or dead. However, in the treatment of bacteria with x-rays, it is possible that the cells classified as dead were actually alive, but no longer capable of fission because of some effect on the reproductive mechanism of the cell.

The methods of detecting variation in this study have inherent weaknesses. For the purpose of the experiments performed, only streaked and poured plates were used to isolate variant forms of colonies. Colonial variations are the easiest to detect, but, they are by no means the only changes that occur, and therefore it is quite possible that many variations were overlooked. On this basis, the studies made on colony changes produced by X-ray treatments can be taken only as an index of activity occurring in the culture. It is probably true, however, that this method of detecting variation is reasonably accurate.

Certain objections might be raised to the interpretations placed on the increase of the dissociation rates, in that the variants were not found in the culture for several days after treatment. Such objections can be dealt with in the following manner. As has been found with higher forms of life, the treatment of a group with x-rays does not result in mutation in all of the individual members. Only a small percentage are actually affected in such a manner that detectable mutations can be found. In the case of x-ray treatment of bacteria, the groups treated consisted of populations of several hundred thousand to one billion individuals per milliliter, as determined by plate counts. If the properties of a small part of one per cent of the population are altered so that a detectable variant occurs, the chance of finding such individuals in random sampling of the population by the streaked and poured plate methods is exceedingly small, since before they can be detected they must increase in numbers. If the variant bacterium is of such a nature as to render it capable of competing with the

rest of the population in the culture, it may take several days for the increase of the variant to occur. If the variations in properties are unfavorable to the organism's further growth, it may die or be overgrown by the rest of the culture and not be found.

The increase in the dissociation rates of the cultures seemed to show that the x-ray energy emitted from both the copper and molybdenum tubes affected the hereditary mechanisms of some of the exposed cells. Not all of the experiments performed led to definite increases in colonial variants, for occasional treatments failed to induce any appreciable effects on the rate of dissociation as determined by colony observations. Likewise, the types of variants that appeared in the cultures of treated washed suspensions were unpredictable. This result is consistent with the findings on higher forms of life.

Observations on the colonial and physiologic characteristics of variants isolated from treated material demonstrated that any of the properties might be changed without being accompanied by alterations in the other properties of the cells. This independence of variation suggests that alterations other than colonial were probably missed, due to a lack of adequate methods of detection.

In the studies on lethal effects, pleomorphism was found to be a rare occurrence. No particular importance can be attached to this, other than the fact that it is another case of hereditary change induced by x-rays. A somewhat similar occurrence was found by Lea, Haines and Coulson (1937) using *Escherichia coli* exposed to x-rays. They found that the cells treated with the rays would occasionally form long filamentous rods. This effect was explained as due to interference with the fission mechanism by x-rays. It is quite possible that the same thing occurred in the treated staphylococci of this study. If Figures 5 and 6 are closely inspected, it can be seen that division and complete separation of the cells have not taken place. The fact that the streptococcus-like form was capable of increase for a period of 24 hours indicates that this pleomorphism is an hereditary alteration of the fission mechanism. Somewhat similar observations were made by Spencer (1935) on the pleomorphism of *Eberthella typhosa* and *Streptococcus scarlatinae* after exposure

to emanations from radium. The cultures of *E. typhosa* contained filamentous rods as described by Lea, *et al.*, for *E. coli*, and *S. scarlatinae* showed formations similar to those of the staphylococci of this report.

In a study of the effects of x-rays on dissociation it is difficult to determine which of the variants are attributable to the action of the rays and which are formed in the natural course of multiplication. For this reason, we do not attribute any of the variants, other than the streptococcus-like form of the treated staphyloccus, to x-ray effects until more can be learned. However, it is apparent in this study that x-rays increased the dissociation rates of several strains of *Staphylococcus aureus* and *Serratia marcescens*.

SUMMARY AND CONCLUSIONS

1. When the logarithms of the per cent of cells surviving x-ray treatment were plotted on semi-logarithmic paper against the time of exposure, a straight line was obtained, indicating that the lethal effects of x-rays are dependent on the number of bacteria present at any given time.

2. The results of the experiments using kilovolts and milliamperes as variables, with time as the constant, demonstrated that the lethal action of x-rays is dependent more on the wave length emitted than on the intensity, once the rays completely bathed the suspension.

3. The short wave lengths (hard rays) used in this study were more'effective in killing *Staphylococcus aureus* than the long wave lengths (soft rays) of the same intensity.

4. X-rays increased the dissociation rates of *Staphylococcus aureus* and *Serratia marcescens*.

5. Actively proliferating cells showed a greater tendency to form dissociants after irradiation than non-proliferating cells. Occasionally streptococcus-like forms resulted from the treatment of *Staphylococcus aureus* with x-rays.

6. The kind or number of variants that result from x-ray treatment could not be predicted. Often no changes were observed.

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THE EFFECT OF HUMAN STRAINS OF HEMOPHILUS INFLUENZAE ON INFLUENZA VIRUS INFECTIONS OF SWINE[1]

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INTRODUCTION

During the 1918 pandemic of influenza two observations were made which have since been interpreted to have some bearing on the possible etiology of epidemic human influenza. The first relates to the bacteriology of the disease, which was thoroughly studied at that time. As a result of these extensive bacteriologic studies, there was an eventual agreement that organisms of the hemophilic group were commonly present in the respiratory tracts of both sick and well persons during the last pandemic period, although there were significant differences in the frequency with which these bacteria were encountered in different regions and in the hands of different workers. The generally high incidence, at that time, of hemophilic organisms in various populations inclined some investigators to conclude that *Hemophilus influenzae* is secondarily important in, or is an integral part of the causation of, pandemic influenza, if not the primary etiologic agent of the disease. On the basis of available facts there is no evidence to indicate that *H. influenzae* is concerned in the etiology of interpandemic epidemic human influenza and its rôle in the pandemic form of the disease must remain an open question.

A second and apparently an unrelated observation which has since been considered significant in relation to the possible etiology

of pandemic influenza was made in 1918 by J. S. Koen, who has been quoted by Dorset, McBryde, and Niles (1922–1923) as observing the appearance of a new disease of swine during the last pandemic of influenza in the midwestern section of the United States. This new epizootic so simulated the prevalent human infection that Koen designated it as "hog Flu." Shope (1931a), Lewis and Shope (1931), and Shope (1931b) examined the problem of "hog flu" or swine influenza and, in a brilliant series of observations, arrived at the conclusion that there are two agents which "act in concert" to produce typical swine influenza. One is a filterable virus and the other is a hemophilic organism, *Hemophilus suis*. On the basis of his observations and because of the many similarities between human and swine influenza, Shope (1931b) suggested that "Pfeiffer's bacillus and a filterable agent (may) act in concert to cause influenza in man." This hypothesis for the etiology of human influenza was given some basis in fact by the isolation, in ferrets, of a filterable agent from typical cases of human influenza by Smith, Andrewes, and Laidlaw (1933) and others. It has further been shown that the viruses of human and swine influenza are antigenically related, (Smith, Andrewes and Laidlaw (1933), Francis (1934–1935) and Shope (1937) and others), but, contrary to the experience with epidemic swine influenza in swine, a bacterial component has not been found essential to the production of either the human or the swine disease in other animal species (Smith, Andrewes, and Laidlaw (1933), Andrewes, Laidlaw, and Smith (1934), Shope (1934, 1935), Francis (1934), Burnett (1935), Stuart-Harris (1936, 1937) and others).

Elkeles (1935) established that young swine are susceptible to the W.S. strain of human influenza and reported that the addition of *H. influenzae* to the injection mixture produces a more severe disease than does the "pure virus" alone. In Elkeles' (1935) only available publication on this subject, the variety or type of influenza bacillus used in his experiments is not designated. Shope and Francis (1936) corroborated Elkeles' observation concerning the susceptibility of swine to laboratory strains of human influenza virus and reported that the presence of *H. suis* in the human virus injection mixture results in a disease which is clin-

ically similar to, and pathologically indistinguishable from, epidemic swine influenza.

The above studies suggest that swine influenza may originally have come from human beings, that human and swine influenza viruses are antigenically related, and that swine are susceptible to at least some strains of human influenza virus.

In addition to the many clinical and epidemiologic similarities between human and swine influenza, and the isolation of related viruses in both diseases, the frequency with which *H. influenzae* has been encountered in the pandemic form of the human disease and in the epidemic form of swine influenza suggests that there may be a similar type of bacterial-virus symbiotic relationship in both diseases. A bacteria-virus "concerted action" has been established in swine influenza. Consequently, it appeared reasonable to test the existence of such a relationship between known human strains of *H. influenzae* and influenza virus strains in swine, since there is some evidence which suggests that human beings and swine react in a similar manner to influenzal infections.

METHODS

1. Animals

Young pigs 8 to 12 weeks old were used in all of the experiments in this study. All animals were observed under isolation conditions for a period before use to ascertain the presence or absence of spontaneous infections and all were bled prior to injection.

2. Virus strains

The V15[2] strain of swine influenza virus was used in the form of either swine-passage or mouse-passage infected lung tissue from freshly sacrificed animals. The swine tissue was highly infective for swine and the titer of the mouse-adapted virus was between 10^{-6} and 10^{-7} MLD per 0.05 ml. in mice. The mouse-adapted PR8[3] strain of human influenza virus which titered

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[2] The V15 strain of swine influenza virus was obtained through the kindness of Dr. R. E. Shope.

[3] The PR8 strain of human influenza virus was generously given us by Dr. Thomas Francis, Jr.

between 10^{-6} and 10^{-7} MLD per 0.05 ml. in mice was used in the human virus experiments.   Five milliliters of a 10 per cent saline suspension of freshly-killed swine-passage or mouse-passage infected lung tissue was used to inject each pig in the experiments to follow.

3. Hemophilus influenzae

Seven human meningeal strains (Pittman type b 1931) of *H. influenzae* were used in these studies.   One strain (no. 62) was a standard stock culture strain of known mouse virulence—the others (nos. 89, 119, 128, 135, 136 and 137) were all used shortly after their isolation from human cases of influenzal meningitis.   Such strains are serologically homogeneous and are in the fully virulent "mucoid" phase of dissociation.

Two freshly isolated human type b strains of *H. influenzae* of non-meningeal origin were used—no. 121 from the respiratory tract of an apparently normal person, and no. 123 from the blood stream of a case of *H. influenzae* meningitis.

Two strains of human respiratory tract origin (nos. 115 and 116) with the dissociative pattern described by Chandler, Fothergill, and Dingle (1939) were used immediately upon their isolation in pure culture.   The growth of 4 to 6 chocolate agar slants of the indicated cultures was taken up in 2.5 ml. of saline and the heavy bacterial suspension was mixed with 5 ml. of the respective virus suspension.   The mixture was inoculated intranasally under deep ether narcosis with the animal held in the upright position during the injection.

After inoculation, the animals were maintained in isolation and observed for symptoms or signs of illness for five days, at the end of which time most of them were sacrificed to ascertain the establishment of the experimental infection.   A few of the animals were allowed to survive and were bled in convalescence to verify the presence of influenza-virus-neutralizing antibodies.   In most instances the infected lung tissue of the sacrificed animals was passed back to mice to verify the presence of the virus in the lesions.   Numerous chocolate agar cultures (12 to 30 on each animal) were taken from various portions of the respiratory tract

of each sacrificed animal and from the nares of surviving animals in order to establish the presence of *H. influenzae* subsequent to their injection.

Histologic sections from each animal were examined to determine the histologic picture of the lesions.

RESULTS

1. V15 swine influenza virus infection in association with human meningeal Pittman type b strains of H. influenzae

In table 1 are shown, in summary, the results obtained by injecting ten swine with swine influenza virus in combination with any one of three human meningeal strains of *H. influenzae.*

TABLE 1

Infections of swine with the V15 strain of swine influenza virus in combination with human strains of H. influenzae

NUMBER OF SWINE INJECTED	HUMAN H. INFLUENZAE STRAIN NUMBER	HUMAN ORIGIN	PITTMAN TYPE	CLINICAL ILLNESS	HEMOPHILUS STRAIN ISOLATED	HISTOLOGY	V15 NEUTRALIZING ANTIBODIES
4*	62	Spinal fluid	b	Minimal*	0*	"Filtrate disease"	−
4	89	Spinal fluid	b	Moderate	+	"Filtrate disease"	+†
2	119	Spinal fluid	b	Minimal	0	"Filtrate disease"	−

* One pig of this group developed typical clinical epidemic swine influenza with the typical histologic picture of this disease and, in addition to the V15 virus, *H. suis* was isolated at post mortem.

= not tested after injection because the animals were sacrificed at the fifth post injection day.

† In one after 6 weeks

It is to be noted that one of the animals in the first group, which received the stock strain no. 62, developed typical epidemic swine influenza clinically; the gross and microscopic pathology was that of epidemic swine influenza as described by Shope (1931a), and *H. suis* was isolated from the bronchi when the animal was sacrificed on the fifth day. The remaining three animals had minimal symptoms, no hemophilic organisms were isolated from the bronchi after infection, and the gross and micro-

scopic pathology was that of the "filtrate" or "pure virus" disease described by Shope (1931b). Two of the second group of animals in this table were injected with a freshly isolated human meningeal strain of *H. influenzae* (no. 89) in combination with the V15 virus, whereas the other two were injected some months later after the bacterial culture had been transferred on artificial media a number of times. The animals injected with the strain at the time of its isolation became moderately ill with a fever between 104 to 105°F., and exhibited symptoms con-

FIG. 1. Bronchial mucosa of an animal killed on the fifth day of epidemic swine influenza (V15 virus + *H. suis*). The hyperplasia and metaplasia of the bronchial epithelium with the loss of its ciliated columnar character is obvious. Although polymorphonuclear cells are present, they are not numerous in this particular section. × 400. Giemsa stain.

sistent with a moderately severe attack of epidemic swine influenza. When these two animals were sacrificed on the fifth day they were found to have an extensive pneumonia which was histologically similar to the "filtrate disease"; but, in place of the proliferation and metaplasia of the bronchial epithelium which is seen in epidemic swine influenza and in the "filtrate disease" (fig. 1), there was a marked necrosis of the bronchial epithelium (fig. 2) which in some instances resulted in complete sloughing of the bronchial epithelium (fig. 3). The necrotic reaction seen in the bronchi of these two swine is interpreted to be the result of some toxic substance from the freshly isolated organism be-

cause there was no such tissue reaction evident in two animals injected with the same bacterial strain in combination with the

FIG. 2. Bronchial mucosa of a swine killed the fifth day after being injected with V15 swine influenza virus in combination with a freshly isolated human meningeal type b strain of *H. influenzae* (no. 89) The tissue was fixed immediately after death. The matting of the cilia, the necrosis, and actual sloughing of the epithelium with no proliferation is evident as is the absence of polymorphonuclear cells. × 460. Giemsa stain.
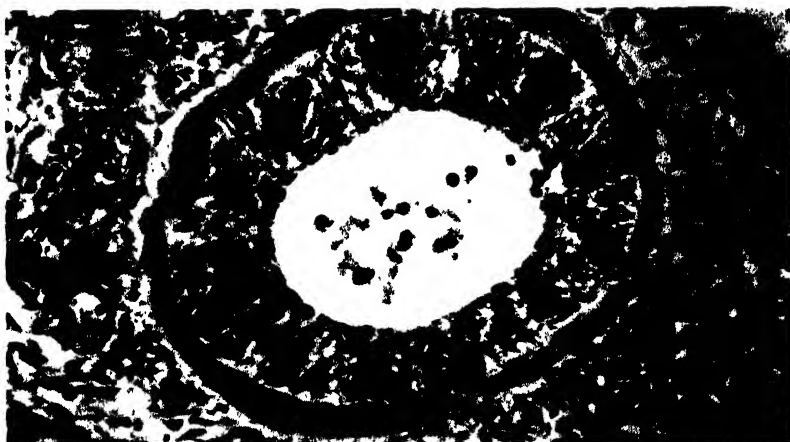
FIG. 3. Small bronchiole of the same animal as in Figure 2 showing a complete loss of the bronchial epithelium. The absence of polymorphonuclear cells is striking. × 200. Giemsa stain.

V15 virus after the organism had been subcultured on artificial media for several months. In the latter instances, only the

"filtrate disease" resulted and the one animal that was allowed to survive to convalescence developed neutralizing antibodies to the V15 virus. This strain (no. 89) of *H. influenzae* is the only one that could be reisolated from the respiratory tracts of injected swine and even this strain could not be established in swine after prolonged growth on artificial media.

The last two swine in table 1 which were injected with another freshly isolated human meningeal type b strain (no. 119) of *H. influenzae* in combination with V15 swine influenza virus developed clinical symptoms and signs consistent with the "filtrate disease" and the lesions were those of the "pure virus" infection. Again in this instance attempts to isolate *H. influenzae* subsequent to injection were unsuccessful.

2. *Human PR8 influenza virus infections of swine in combination with human strains of H. influenzae*

The results of injecting 19 swine with the PR8 mouse-adapted strain of human influenza virus in combination with any one of a number of human strains of *H. influenzae* are shown in table 2. Examination of the data in the top section of this table reveals that 11 swine were injected with any one of 6 human meningeal type b strains of *H. influenzae*, all of which were used when freshly isolated except the stock strain no. 62, and strain no. 89, which was used only after prolonged growth on artificial media.

An examination of the several columns in the upper portion of this table reveals that in all cases the clinical illness was mild or not detectable; in no instance were hemophilic organisms isolated subsequent to injection, and the histology of the minimal lesions in the sacrificed animals was that of a "pure virus" infection, with one exception in which there were no lesions. (In this instance PR8 neutralizing antibodies were detectable in the blood prior to the experimental injection.) The presence of virus in the lung lesions of the sacrificed animals was checked in most instances by passing lung tissue of infected swine back to mice. Furthermore, the sera of the animals that were allowed to convalesce were tested for the presence of PR8 human influenza virus-neutralizing antibodies which were found to be present in all of the animals permitted to survive.

TABLE 2

Infections of swine with the PR8 strain of human influenza virus in combination with human strains of H. influenzae

NUMBER OF SWINE INJECTED	HUMAN H. INFLUENZAE STRAIN NUMBER	HUMAN ORIGIN	PITTMAN TYPE	CLINICAL RATINGS	HEMOPHILUS STRAIN ISOLATED	ETIOLOGY	PR8 NEUTRALIZING ANTIBODIES
5	62	Spinal fluid	Type b	Minimal	0	"Filtrate disease"	+ in 2 after 6 weeks
2	89	Spinal fluid	Type b	Minimal	0	"Filtrate disease"	+ in 1 after 6 weeks
1	128	Spinal fluid	Type b	Mild	0	"Filtrate disease"	—
1	135	Spinal fluid	Type b	None	0	No lesions	+ before injection
1	136	Spinal fluid	Type b	Minimal	0	Not examined	+ after 5 weeks
1	137	Spinal fluid	Type b	Mild	0	"Filtrate disease"	—
2	123	Blood stream	Type b	None	0	Epidemic swine influenza	—
2	121	Respiratory tract	Type b	None	0	"Filtrate disease"	—
2	115	Respiratory tract	"Rough"	None	0	"Filtrate disease"	—
2	116	Respiratory tract	"Rough"	Minimal	0	"Filtrate disease"	+ in 1 after infection

— = not tested after injection because the animals were sacrificed at the fifth post injection day.

In addition to the group injected with meningeal strains of *H. influenzae* in the upper section of table 2, two swine were injected with a type b strain (no. 123) of *H. influenzae* isolated from the blood stream of a child and two were injected with a type b strain (no. 121) isolated from the respiratory tract of a normal individual—each in combination with the PR8 virus. In the former instance no clinical illness resulted, but when the animals were sacrificed on the fifth day there were fairly large areas of consolidation and atelectasis, with very little exudate in the bronchi; no hemophilic organisms were isolated from the bronchi or lung parenchyma in either instance, but the histologic pathology revealed a much more marked polymorphonuclear cell reaction than is seen in the "filtrate" of "pure virus" disease. The two animals injected with the type b strain (no. 121) from the respiratory tract had no symptoms or signs of illness, the gross lesions were minimal, no hemophilic organisms could be isolated from the respiratory tract, and the microscopic lesions resembled the "filtrate disease."

The four swine in the lower division of table 2 which were injected with the two respiratory strains of *H. influenzae* (nos. 115 and 116) in combination with the PR8 virus had no clinical illness subsequent to injection, the gross lesions were minimal, the organisms were not isolated after injection, and the histologic lesions in the sacrificed animals were consistent with the "filtrate disease." One animal that was allowed to convalesce developed neutralizing antibodies to the PR8 virus, thereby demonstrating that the PR8 virus was able to establish itself in contrast to the apparent inability of human strains of *H. influenzae* to establish themselves in the swine respiratory tract.

DISCUSSION

The results of the first group of experiments shown in table 1 fail to reveal any marked symbiotic or synergistic action between V15 swine influenza virus and the three human meningeal type b strains of *Hemophilus influenzae* studied, and in all instances the histopathology resembled that of the "filtrate" or "pure virus"

disease. The one exception, with the newly isolated strain (no. 89), is explained on the basis of a possible toxic substance, because this strain had no such action with either the V15 or the PR8 virus after it had been grown on artificial media for sometime. With this exception the results suggest that in most instances human meningeal strains of *H. influenzae* cannot establish and maintain themselves in the swine respiratory tract.

The results in the second group of experiments in table 2 also fail to reveal any broad concerted or synergistic action between either the meningeal type b strains, or the human respiratory strains of *H. influenzae* studied, and the PR8 strain of human influenza virus in swine. The bacteriologic results in the second table corroborate the above impression that human strains of *H. influenzae* usually fail to establish and maintain themselves in the swine respiratory tract and suggest a biologic difference between human and swine strains of *H. influenzae*.

It must remain for future work to determine whether or not other human strains of *H. influenzae* can act symbiotically or in concert with these or other strains of influenza virus in either swine or man. In any case, it is evident that there is no broad synergistic action between human hemophilus strains and these two well established virus strains in swine, which again raises the question whether or not there is an *H. influenzae*-influenza virus symbiotic relationship in human influenza of the nature reported in swine influenza.

SUMMARY

The possible existence of a concerted or synergistic action between known human strains of *Hemophilus influenzae* and two influenza virus strains has been investigated in swine.

In no instance has a significant symbiotic action been demonstrated between the bacterial and the virus strains studied.

The inability of human strains of *H. influenzae* to establish themselves in the respiratory tract of swine suggests a possible biologic difference between human and swine strains of *H. influenzae*.

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THE INHIBITION OF BACTERIOPHAGY BY CHOLES-
TEROL AND BY BACTERIAL AND NON-BACTERIAL
PHOSPHOLIPIDS

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The mechanism of bacteriophagy is not understood. A variety of hypotheses have been proposed, based on assumptions ranging from a mechanical penetration of the cell (d'Herelle, 1926) to a union dependent on antigenic components (Burnet, 1930; Levine *et al.*, 1933). White (1936) has suggested that cellular lipids may be involved in bacteriophagy. The present study was designed to investigate this hypothesis. Accordingly, we have examined the lipids of several strains of *Escherichia*. Early in our investigation, it became clear that these lipids were almost entirely phospholipid in nature (Williams, Bloor and Sandholzer, 1939). Thus, it became essential to determine whether or not these bacterial phospholipids—and, for comparison, cholesterol and certain non-bacterial phospholipids—inhibited bacteriophagy. Our findings with these substances are given in the present report.

Levine and Frisch (1933) were the first to note that certain constituents of the bacterial cell inhibited bacteriophagy. They showed that the specific polysaccharide of susceptible strains of *Salmonella* inhibited the homologous bacteriophage specifically, a finding confirmed by Burnet (1934). Two years later, Levin and Lominski (1936) found that various lecithins slightly inhibited a

bacteriophage active against certain strains of *Staphylococcus*. Later, in the same year, they reported that cholesterol had a similar action. Further evidence of an inhibition of bacteriophagy by phospholipids was presented by White (1936), who showed that a bacteriophage active against certain strains of *Vibrio cholerae* could be inhibited by an acetone-insoluble lipid derived from either sensitive or resistant strains. Before inhibition was evidenced, however, it was necessary for his suspensions to be very dense. These few reports concerning the inhibition of bacteriophagy by lipids seem to indicate that the reaction is non-specific in character.

MATERIALS AND METHODS

Bacterial strains. The 3 bacterial strains (214, 234 and 239-1) employed in the present study belonged to the genus *Escherichia*. Strains 234 and 239-1 were typically *Escherichia coli* var. *communior* and both were susceptible to lysis by the bacteriophage employed. On the other hand, 214 was a strain of *Escherichia coli* which was resistant to the bacteriophage. The bacteriophage was not regenerated following the lysis of 239-1. Preparations of phospholipid were made from each strain.

Bacteriophage. Bacteriophage C13, shown to be active against coliform organisms by Burnet (1930), was used throughout. Bacteriophagy was studied quantitatively by the plaque-count method in semi-solid agar. In each experiment, to test for the inhibition of bacteriophagy, 2 sets of serial dilutions of bacteriophage were made in peptone water plus the test substance and 2 in peptone water alone. After these dilutions had been incubated at 37°C. for 24 hours, 0.5 ml. of a 24-hour peptone-water culture of the test organism was added to each tube of diluted bacteriophage. Plates were then poured in triplicate of semi-solid agar with 1 ml. aliquots from each tube of peptone-water culture. These plates were incubated at 37°C. for 24 hours, when the number of plaques appearing on each plate was counted.

Culture media. Two culture media were employed in the present study, peptone water and semi-solid nutrient agar.[1]

PREPARATION OF TEST SUBSTANCES

A. Non-bacterial phospholipids

Lecithin. Crude lecithin from soybean was dissolved in petroleum ether and purified by repeated precipitations with acetone. After the last precipitation, it was redissolved in petroleum ether, and aliquots of this solution were used to determine the concentration of lecithin. This measurement was made by warming the samples to remove the petroleum ether and then drying them in a rapidly moving current of air to provide residues for weighing.

In preparing the lecithin for testing against bacteriophage, the desired amount of the solution in petroleum ether was pipetted into a sterile 300-ml. Erlenmeyer flask containing sterile glass beads. The solution was then evaporated nearly to dryness and 1 ml. of alcohol added to the moist residue. After warming the solution, the required volume of sterile peptone water was added slowly while the flask was being constantly agitated. This procedure produced a homogeneous, cream-colored suspension which was immediately employed in the tests. Fresh suspensions were prepared for each experiment.

Cephalin. The alcohol-insoluble component of the phospholipid fraction from beef liver and from sheep brain was used as cephalin. The preparations were purified, assayed and employed as described for lecithin.

[1] Peptone water:

Peptone (bacto)...........................	20 grams
NaCl.......................................	5 grams
Water..	1000 ml.

Reaction adjusted to pH 7.4 to 7.6

Semi-solid nutrient agar:

Peptone (bacto).....	5 grams
Beef extract (bacto).........	3 grams
Agar (bacto).................................	5 grams
Water..........	1000 ml.

Reaction adjusted to pH 7.4 to 7.6

Phospholipid from plasma.   A sample of pure, mixed phospholipid from the plasma of a dog was dissolved in moist ether, centrifuged, and the ether layer siphoned into a volumetric flask. Aliquots of the ether layer were evaporated to dryness and weighed.   A suspension of the phospholipid was prepared as described under lecithin.

Sphingomyelin.   Sphingomyelin from sheep brain, provided as a white powder,[2] was weighed directly in a sterile flask and the required amount of sterile peptone water added.   The mixture was shaken at intervals during a period of 6 hours in order to suspend the phospholipid completely.

Cholesterol.   Cholesterol from gall stones was dissolved in hot petroleum ether and allowed to crystallize in the cold from this solvent.   The crystals were filtered off, washed with cold petroleum ether and then redissolved in hot acetone.   The cholesterol was then recrystallized 3 times from cold acetone solution and dried in the air.   It melted at 146.2°C. (uncorrected).   The reported melting point for cholesterol is 147.5°C.

For the tests with bacteriophage, a weighed sample of cholesterol was dissolved in 5 ml. of hot alcohol in a sterile flask.   The peptone water was added slowly, the flask being rotated during the addition.

B. Bacterial phospholipids

Bacteria grown in 1-per cent peptone water for 48 hours were harvested by means of a Sharples centrifuge.   The phospholipid was extracted by methods described previously (Williams, Bloor and Sandholzer, 1939) and was purified by 3 precipitations with acetone from a petroleum ether solution.   The petroleum-ether solution of purified phospholipid was made up to 50 ml. in a volumetric flask.   Two aliquots of 1 ml. each were taken and the amount of phospholipid determined oxidatively (Bloor, 1928). The remaining 48 ml. were evaporated to dryness in a sterile Erlenmeyer flask and 1 ml. of alcohol added while the flask was still on the steam bath.   The calculated volume of peptone water

[2] We are indebted to Mr. J. D. Taylor for the samples of sphingomyelin used in our work.

was added to the warm alcoholic solution, the flask being shaken during the addition.   The suspension was used immediately.

RESULTS

The influence of the non-bacterial phospholipids on bacteriophage C13 is shown in tables 1, 2 and 3, and that of the bacterial

TABLE 1

Influence of lecithin, cephalin and sphingomyelin on bacteriophage C13 acting on Escherichia coli var. communior 234

PHOSPHOLIPID EMPLOYED	CONCEN-TRATION	AVERAGE PLAQUE-COUNT WITH STANDARD DEVIATION FOR 6 PLATES OF EACH DILUTION OF BACTERIOPHAGE				
		10⁻¹	10⁻²	10⁻³	10⁻⁴	10⁻⁵
	mgm. per ml.					
Lecithin (soybean)..	10	S	S	T.N.	35 ± 4.8	4 ± 1.0
Control.............	—	S	S	T.N.	34 ± 6.0	6 ± 1.9
Cephalin (beef liver)	10	T.N.	21 ± 4.1	6 ± 2.3	0	0
Control.............	—	S	74 ± 6.2	35 ± 6.2	0	0
Cephalin (beef liver)	2	S	495 ± 71.0	429 ± 64.8	9 ± 2.6	8 ± 2.0
Control.............	—	S	T.N.	T.N.	56 ± 5.3	66 ± 18.0
Cephalin (sheep brain)...........	2	T.N.	T.N.	183 ± 14.6	20 ± 7.2	4 ± 1.0
Control.............	—	S	T.N.	T.N.	107 ± 22.4	22 ± 4.4
Sphingomyelin (sheep brain)....	2.15	—	T.N.	T.N.	24 ± 2.9	23 ± 2.9
Control.............	—	—	T.N.	T.N.	34 ± 8.1	36 ± 4.9
Lecithin + cephalin.	10 + 2	—	—	151 ± 17	3 ± 2.7	2 ± 1.0
Lecithin + cephalin.	5 + 2	—	—	264 ± 32	4 ± 0.1	6 ± 1.4
Control.............	—	—	—	T.N.	37 ± 3.2	33 ± 2.8

S, "sterile" plate, *i.e.*, no visible growth of bacteria; T.N., plaques too numerous to count; 0, no plaques, *i.e.*, no evidence of bacteriophage; —, experiment not performed.

phospholipids is recorded in table 4.   The data are presented in terms of plaque-counts, with the standard deviation given in each instance.   Summaries of the data analyzed statistically are given in tables 5 and 6.

The actions of the monoaminophospholipids, lecithin and

TABLE 2

Influence of lecithin and cephalin on bacteriophage C13 acting on Escherichia coli var. communior 239-1

PHOSPHOLIPID EMPLOYED	CON-CEN-TRA-TION	AVERAGE PLAQUE-COUNT WITH STANDARD DEVIATION FOR 6 PLATES OF EACH DILUTION OF BACTERIOPHAGE				
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
	mgm. per ml.					
Lecithin (soybean).....	10	T.N.	T.N.	89 ±22.6	13 ±2.2	1 ±1
Control................	—	T.N.	T.N.	82 ±16.0	12 ±3.2	2 ±1
Cephalin (beef liver)...	10	148 ±16.8	14 ± 5.6	2 ± 0	0	0
Control................	—	370 ±38.4	56 ± 8.4	6 ± 3.1	0	0
Cephalin (beef liver)...	2	T.N.	134 ±24.7	17 ± 2.0	3 ±1.1	0
Control................	—	T.N.	T.N.	86 ±13.6	9 ±2.0	0

Symbols as in table 1.

TABLE 3

Influence of cholesterol and phospholipid from plasma on bacteriophage C13

SUBSTANCE EMPLOYED	CON-CEN-TRA-TION	NUM-BER OF TEST ORGAN-ISM	AVERAGE PLAQUE-COUNT WITH STANDARD DEVIATION FOR 6 PLATES OF EACH DILUTION OF BACTERIOPHAGE				
			10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
	mgm. per ml.						
Cholesterol (gall stones)	10	234	S	152 ±24.9	69 ± 4.5	2 ± 1.4	0
Control.........	—	234	S	338 ±38.6	130 ±18.2	7 ± 1.7	0
Cholesterol (gall stones).........	10	239-1	T.N.	63 ±16.4	21 ± 4.8	1 ± 0	0
Control.........	—	239-1	S	179 ±37.4	70 ±12.6	4 ± 1.7	0
Plasma phospho-lipid...........	5	234	S	S	T.N.	51 ±10.8	50 ±12.1
Control.........	—	234	S	T.N.	T.N.	22 ± 3.2	34 ± 6.7
Plasma phospho-lipid...........	10	234	T.N.	430 ±54.1	6 ± 2.6	8 ± 3.0	0
Control.........	—	234	T.N.	525 ±47.7	15 ± 2.8	12 ± 2.8	0

Symbols as in table 1.

cephalin, and of the diaminophospholipid, sphingomyelin, on the bacteriophage, when strain 234 was the test organism, are shown

in table 1.   Lecithin apparently did not inhibit the bacteriophage, whereas cephalin showed a definite retarding action, even when used in a concentration of 0.2 per cent.   This action was still evident after lecithin had been mixed with cephalin in the proportion of 5 to 1.   Sphingomyelin had no inhibitory action.

TABLE 4

Influence of phospholipids from three strains of enteric bacilli on bacteriophage C13

SOURCE OF PHOSPHOLIPID	NUMBER OF TEST ORGANISM	AVERAGE PLAQUE-COUNT WITH STANDARD DEVIATION FOR 6 PLATES OF EACH DILUTION OF BACTERIOPHAGE				
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Escherichia coli var. communior 234....	234	—	15 ± 5.8	3 ± 1.7	0	0
Control............	234	—	8	T.N.	114 ± 6.7	15 ± 5.8
Escherichia coli var. communior 234....	234	8	T.N.	82 ± 7.8	9 ± 2.3	0
Control............	234	8	8	T.N.	101 ± 16.4	13 ± 1.6
Escherichia coli var. communior 239-1...	239-1	423 ± 6.4	19 ± 3.5	2 ± 1.6	0	0
Control............	239-1	1130 ± 218.0	106 ± 14.4	19 ± 6.8	1 ± 1.1	0
Escherichia coli var. communior 239-1...	239-1	0	0	0	0	—
Control............	239-1	T.N.	349 ± 77.4	54 ± 11.8	6 ± 2.6	—
Escherichia coli 214..	234	0	0	0	0	—
Control............	234	8	T.N.	T.N.	351 ± 53.2	—
Escherichia coli 214..	239-1	158 ± 20.2	6 ± 2.0	0	0	—
Control............	239-1	T.N.	250 ± 18.1	32 ± 5.6	4 ± 2.0	—

Symbols as in table 1.

The findings with lecithin and cephalin, when strain 239-1 was used as the test organism, are shown in table 2.   The data are not as complete as those for strain 234, but the same effect was observed, *i.e.*, lecithin failed to inhibit bacteriophagy but cephalin did.   The combined action of these substances was not studied with strain 239-1.

The action of cholesterol and phospholipid from dog plasma is

shown in table 3. Regardless of the test organism employed or the dilution of the bacteriophage, cholesterol produced some inhibition when present in a concentration of 1 per cent. This

TABLE 5

Statistical analysis of the influence of the lipids of three strains of enteric bacilli, cephalin and cholesterol on bacteriophage C13

SOURCE OF TEST SUBSTANCE	NUMBER OF TEST ORGANISM	CONCENTRATION	PER CENT INHIBITION AND $\bar{x}/\sigma$ DIFF.* AT VARIOUS DILUTIONS OF BACTERIOPHAGE				
			10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
		mgm. per ml.					
Escherichia coli var. communior 234	234	2	—	—	—	100.0†	100.0
			—	—	—	17.0	2.8
Escherichia coli var. communior 234	234	2	—	—	—	91.1	100.0
			—	—	—	5.6	8.6
Escherichia coli var. communior 239-1	239-1	2	62.6	82.1	88.9	100.0	—
			3.4	5.8	2.5	1.0	—
Escherichia coli var. communior 239-1	239-1	2	—	100.0	100.0	100.0	—
			—	4.5	4.5	2.3	—
Escherichia coli 214	234	2	—	—	—	—	100.0
			—	—	—	—	6.5
Escherichia coli 214	239-1	2	—	97.6	100.0	100.0	—
			—	13.4	5.7	2.0	—
Cephalin (beef liver)	234	10	—	71.7	82.9	—	—
			—	7.1	4.3	—	—
Cephalin (beef liver)	234	2	—	—	—	84.0	87.9
			—	—	—	8.1	3.2
Cephalin (sheep brain)	234	2	—	—	—	81.4	81.9
			—	—	—	3.6	4.0
Cephalin (beef liver)	239-1	10	60.0	75.0	66.7	—	—
			5.2	4.2	1.3	—	—
Cephalin (beef liver)	239-1	2	—	—	80.3	66.7	—
			—	—	5.0	2.6	—
Cholesterol (gall stones)	234	10	—	55.1	47.0	73.0	—
			—	4.0	3.2	1.8	—
Cholesterol (gall stones)	239-1	10	—	64.9	70.0	75.0	—
			—	2.8	3.6	1.8	—

* $\bar{x}/\sigma$ diff. = deviation from arithmetic mean ÷ standard error of the difference.

† Per cent inhibition is given in each instance as the upper figure, the $\bar{x}/\sigma$ diff. as the lower.

finding is in accord with Levin and Lominski (1936). The phospholipid from plasma possessed no inhibitory properties for bacteriophage C13.

The inhibitory influence of the bacterial phospholipids is shown in table 4. It can be seen that these substances were as active in inhibiting bacteriophagy as the non-bacterial phospholipids. It made little difference, furthermore, whether or not the organism from which the phospholipid was derived was susceptible to the bacteriophage. Thus, the phospholipid from resistant strain 214 was as effective in inhibiting bacteriophagy

TABLE 6

Statistical analysis of the influence of lecithin, sphingomyelin and phospholipid from plasma on bacteriophage C13

SOURCE OF TEST SUBSTANCE	NUMBER OF TEST ORGANISMS	CONCENTRATION	PER CENT INHIBITION AND $\bar{x}/\sigma$ DIFF.[*] AT VARIOUS DILUTIONS OF BACTERIOPHAGE				
			10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
		mgm. per ml.					
Lecithin (soybean)	234	10	—	—	—	−2.0[†]	33.4
			—	—	—	0.13	0.95
Lecithin (soybean)	239-1	10	—	—	−8.0	−8.0	50.0
			—	—	0.26	0.25	1.0
Lecithin plus cephalin	234	5 to 1 ratio	—	—	—	91.9	93.3
			—	—	—	8.0	10.6
Lecithin plus cephalin	239-1	5 to 2 ratio	—	—	—	89.2	81.9
			—	—	—	10.3	8.7
Sphingomyelin (sheep brain)	234	2.15	—	—	—	29.5	36.2
			—	—	—	1.2	2.1
Plasma phospholipid	234	5	—	—	—	−131.0	−47.0
			—	—	—	2.6	1.2
Plasma phospholipid	234	10	—	18.1	60.0	35.0	—
			—	1.3	2.3	0.97	—

[*] $\bar{x}/\sigma$ diff. = deviation from arithmetic mean ÷ standard error of the difference.

[†] Per cent inhibition is given in each instance as the upper figure, the $\bar{x}/\sigma$ diff. as the lower.

as that from either of the sensitive strains. This finding is in agreement with White (1936).

The per cent of inhibition of bacteriophagy and the statistical significance of the difference between the average values obtained for the control and test substances—which could be calculated, obviously, only when the number of plaques could be counted in each case—are recorded in tables 5 and 6. The formula used to calculate the latter value was $\bar{x}/\sigma$ diff., in which $\bar{x}$ equals the

difference between the arithmetic means and σ diff. equals the standard error of the difference (Dunn, 1929). When the value for this ratio is 2 or more, the data are usually regarded as significant.

From table 6 it is clear that neither lecithin, sphingomyelin nor plasma phospholipid had an inhibitory influence on bacteriophagy, the average values for $\bar{x}/\sigma$ diff. being less than 2. The inhibitory effect of bacterial phospholipids, cephalin and cholesterol is well shown in table 5. The average per cent inhibition for the bacterial phospholipids was 94; for cephalin, 77; and for cholesterol, 64. When each series of values was calculated for probable error, it was found that the amount of inhibition was about the same for the three different substances. The average values for $\bar{x}/\sigma$ diff. considerably exceeded 2. Whether or not the bacterial phospholipids had been derived from resistant or susceptible strains, the inhibitory action was equally marked. Both preparations of cephalin gave inhibitions of the same magnitude.

DISCUSSION

The data given in the present study show that cholesterol, cephalin and bacterial phospholipids inhibit bacteriophagy, while lecithin, sphingomyelin and plasma phospholipid do not. The mechanism of this inhibition is not clear, but it is probably concerned with the difference in chemical composition of the substances studied. Cephalin, for example, is more acidic in solution than lecithin, and cholesterol differs from both of these substances in that it is a secondary alcohol. The bacterial phospholipids are also monoamino in character. The content of lecithin and cephalin in these phospholipids has not been determined. Further investigations are under way at present.

The inhibition of bacteriophagy with bacterial phospholipids is independent of the susceptibility or resistance of the organism, and is therefore not specific in the sense of an antigen-antibody union. White (1936) had already observed this non-specificity of inhibition with the lipids of *Vibrio cholerae*. Thus, the phospholipid fractions of these organisms, in contrast to the polysac-

charide fractions obtained by Levine and Frisch from strains of *Salmonella*, are not specific inhibitors of bacteriophagy in an immunological sense.

SUMMARY

1. Observations on the inhibition of bacteriophage C13 by cholesterol and by bacterial and various non-bacterial phospholipids are presented.

2. Cephalin, cholesterol and the bacterial phospholipids inhibited the bacteriophage when susceptible strains of *Escherichia coli* var. *communior* were used as the test organisms.

3. Lecithin, sphingomyelin and plasma phospholipid had no inhibitory action.

4. Phospholipid from a strain of bacteria resistant to the bacteriophage was equally as active as that from a susceptible strain.

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STUDIES WITH BACTERIOPHAGES ACTIVE AGAINST MUCOID STRAINS OF BACTERIA[1]

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Bacteriophages acting on mucoid strains of *Klebsiella*, *Aerobacter*, and *Escherichia* are strikingly different from most of the other lytic agents in two important respects (1) the plaques which are produced have an unusual and distinct morphology; and (2) they are highly type-specific in their action, this type-specificity closely paralleling their serological behavior.

The plaques, which appear when appropriate dilutions of phage are layered over previously inoculated Savita agar plates of a mucoid strain, consist of a clear area (the true plaque) varying in size from pin point to four millimeters in diameter, which is surrounded by a translucent or "ground glass" band (hereafter designated as "the zone"). These zones vary in size from one to five millimeters upon first appearance, and with further incubation the width of the zone increases until it may cover the entire plate culture (see figures 1, 2 and 3). The organisms found in the zone are not dead; they have, however, been deprived of their capsules and are avirulent. Bacteriophage can invariably be isolated from all parts of the zone as well as from the plaque itself.

Of secondary importance, is the possibility that phages may be very useful in classifying members of the genus *Klebsiella* into their respective types– since, with the limited number of strains that we have employed, the phages acting on *Klebsiella pneumoniae* Type A are restricted in their action toward these strains, having no lytic effect on "S" cultures of Types B and C. Phages

acting on Type B strains have no demonstrable action on Type A and C strains. Phages lysing the two Type C strains are also specific.

FIG. 1. PHAGE AMag ACTING ON KLEBSIELLA PNEUMONIAE TYPE A
Appearance of plaque with surrounding zone. Incubated at 35°C. for 18 hours.
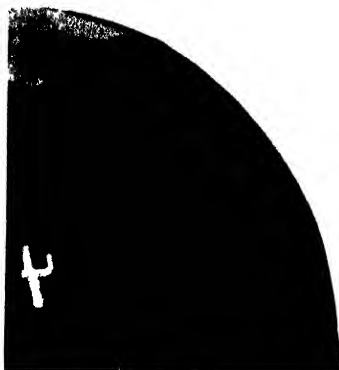
FIG. 2. APPEARANCE OF THE SAME PLAQUE AFTER INCUBATION FOR ANOTHER TWENTY FOUR HOURS AT 35°C.
Note increase in the size of the zone, but no increase in the size of the plaque.

Bacteriophages active against *Klebsiella* organisms were first reported by Caublot (1924), and by Hadley (1925). Both of these investigators mentioned that secondary cultures were devoid of capsule and lacked pathogenicity. Kimura (1925) produced mucoid variants of *Escherichia coli* which tended to be phage-

resistant, and he postulated that the presence of mucoid material may serve to prevent phage from attacking the bacterial cell. Others have, through the action of phage, produced mucoid variants, which themselves were lysogenic (Bordet and Ciuca, 1921; Gratia, 1921; d'Herelle and Beecroft, 1932). It has been assumed that the presence of capsule probably endows the organism with a protective coating that prevents bacteriophage from coming in contact with the cell surface.   However, most strains of bacteria hat produce capsule do so in the "S" state of their development,

FIG. 3. ACTION OF A BACTERIOPHAGE ACTING ON A KLEBSIELLA PNEUMONIAE TYPE B STRAIN

All the plaques surrounded by zones, which have begun to coalesce.   Incubation at 35°C. for 18 hours.

and it is precisely in this phase that organisms are usually most susceptible to bacteriophagy.

All of the phages used in this study were isolated from sewage. Briefly, the method consists in adding to 900 ml. of sewage water—made slightly alkaline by the addition of NaOH— 100 ml. of ten-times concentrated Savita broth, pH 7.5.   This mixture was then divided into a series of Erlenmeyer flasks—approximately 200 ml. per flask—and to each portion was added 10 ml. of an 18-hour broth culture for which a phage was being sought. After incubation at 35°C. for 18 to 22 hours a generous amount was filtered through an L₅ Chamberland candle.   The filtrate was then tested for lytic action against homologous and other cultures.   This method was generally successful whereas when the

sewage was filtered directly or incubated after adding only broth without culture, any phage that was isolated was a weak one.

The medium employed throughout this study was Savita broth and Savita agar (1 per cent).

PHAGES ACTING ON AEROBACTER AEROGENES STRAINS

Seven strains of bacteria belonging to this group were employed. Six phages isolated for strain Soil ＃3 and one phage isolated for strain AW1 were tested for activity against our *Aerobacter* cultures as well as organisms belonging to the genus *Klebsiella*. All seven strains of *Aerobacter* were Voges-Proskauer positive; failed to produce indole and when tested by an India ink method,

TABLE 1

Activity of phages against Aerobacter aerogenes strains

PHAGE	AER. S ＃3	AER. ＃11	AER. AW1	AER. AF11	AER. M. STATE	AER. BYRD	AER. RVS.
Eg S3/37	4+	0	0	3+	0	0	0
Eg S3 1/38...	4+	2+	0	3+	0	1+	3+
Eg S3 5/19	4+	0	0	2+	4+	0	0
Eg S3 111 . .	4+	0	0	1+	0	0	0
Eg S3 111M	4+	0	0	3+	3+	0	3+
Eg S3 11/21 . .	4+	0	0	1+	2+	0	0
Eg AW1	0	0	4+	0	0	0	0

4+, complete lysis; 3+, presence of a few colonies; 2+, plaque with many colonies; 1+, plaque covered with colonies; 0, no observable action.

all seven strains showed capsules. Strain S ＃3 injected intraperitoneally into white mice killed them regularly within a period of 10 to 18 hours. The lytic effect of the various phages on the *Aerobacter* strains, when tested by the "cross test" (Asheshov, 1933) is set forth in Table 1.

All of the S3 phages also lysed a strain of *Klebiella rhinoscleromatis* (R-243) and a few strains of *Klebsiella pneumoniae* Type B. None of these phages attacked strains belonging to Types A and C. The results listed above are those obtained when the *primary* sewage filtrate is used. When one of these filtrates is passed serially against strain S ＃3 it loses its activity for all other organisms except the homologous one. This is due, undoubtedly, to the

dropping out of phage types, a phenomenon which is commonly encountered in developing sewage filtrates against single strains of enteric organisms.   In contrast to the polyvalency of the primary filtrates isolated for *Aerobacter* strain S #3 the phage isolated for *Aerobacter* strain AW1 lysed only this strain and a culture of *Klebsiella granulomatis*.

The sewage filtrates containing active lytic principle for strain S #3 when plated against this strain on Savita agar gave rise to several different kinds of plaques.   They varied from pin-point in size with an accompanying zone 1 to 3 mm. in diameter, to plaques having a diameter of 3.5 mm. encircled by a zone often extending 5 mm. from the edge of the plaque.   Plaques without zones were also observed.   Thirteen pure-line phages, using the technique described in an earlier paper (Rakieten, *et al*, 1937) were isolated from five of the sewage filtrates acting on strain S #3.   While they were originally classified as pure-line on the basis of plaque morphology, it was found on further study that these thirteen types fell into six groups based on activity toward secondary cultures.   This latter evidence signifies that in sewage filtrates certain phage types keep recurring.   This is particularly interesting since in some instances several months elapsed between our attempts to isolate phages from sewage against this culture.

It became evident early in this study that the zones surrounding the plaques grew larger and eventually coalesced, but that the plaques, regardless of the length of time that the plates were incubated, did not increase perceptibly after the first twenty-four-hour incubation period.   The increase in the zone occurs as regularly at room temperature as at incubator temperatures, and steadily progresses day by day until the entire plate culture has been acted upon.   This consists chiefly in the conversion of the mucoid growth into one that is translucent.   From the plaque itself, as well as from the culture within the zone, phage could be isolated.   It is important to keep in mind that the zone progressively spreads through a culture which is many days old; but if one places phage on a twenty-four-hour old plate culture, in order to see whether any action can be observed, no change in the culture takes place where the phage has been deposited.   When

phage is plated on non-mucoid susceptible variants of *Aerobacter* strains, plaques develop, but these plaques are not surrounded by a zone.

PHAGES ACTING ON KLEBSIELLA PNEUMONIAE TYPE B

All of the strains in this group are pathogenic for mice; all are indole-negative and are agglutinated by Type B anti-serum. They possess capsules and grow luxuriantly on Savita agar and in Savita broth. These cultures with the exception of strain EGS (from Dr. Julianelle) were from the collection of Miss Osterman, Department of Bacteriology, Yale University. Strain EGS was

TABLE 2

Action of sewage filtrates isolated against K. pneumoniae Type B strain Egs, on cross test

Klebsiella pneumoniae

PHAGE	TYPE B									TYPE A				TYPE C F10	AERO-BCT.		KL. RHINOSCL
	Egs	Caroli	13	7	2328	17	coli	114	15	So	F5	Hlp.	Mag.		S#3	M. state	
B.	4+	4+	4+	4+	4+	4+	3+	4+	4+	0	0	0	0	0	2+	1+	2+
B 11/24.	4+	4+	4+	3+	4+	4+	4+	4+	4+	0	0	0	0	0	0	4+	4+
B 12/20 . . .	4+	4+	4+	4+	4+	4+	4+	4+	2+	0	0	0	0	0	0	4+	4+
B (x)	4+	4+	4+	4+	4+	4+	4+	4+	4+	0	0	0	0	0	2+	1+	4+
B 10/21	4+	4+	4+	2+	4+	4+	3+	4+	4+	0	0	0	0	0	0	3+	4+

considered as the type strain in this group, and five phages have been isolated against this strain from sewage by the use of the same method employed for *Aerobacter* phages. The results listed in table 2 indicate the action of these phages on homologous type strains as well as on others.

An inspection of the results listed above makes it quite clear that five different sewage filtrates isolated against one strain of Type B (EGS) exhibit strong lytic action on eight other Type B strains. No action is noted against Type A and two Type C strains. Three strains of *Aerobacter* were lysed by these filtrates (the two listed above) and strain Byrd. *Klebsiella rhinoscleromatis* strain R-243 is also susceptible to these filtrates. The

polyvalency of these filtrates is not lost when they are serially passed against strain EGS, as contrasted with what takes place when sewage filtrates active against *Aerobacter* strains are serially passed against *Aerobacter* strain S ✳3.

All of these phages produce plaques varying from 1 to 4 mm. in diameter, surrounded by zones extending 2 to 5 mm. beyond the edge of the plaque, when they are plated on smooth, mucoid Type B cultures. The plaques themselves, on continued incubation, remain fixed in size, but the zones continue to spread throughout the entire plate culture, converting the mucoid growth to one that is vitreous in appearance. This vitreous growth carries phage, does not possess capsule, and no longer is pathogenic for white mice. If a plate contains a large number of plaques when observed after 18 hours incubation, the mucoid portion not touched as yet by phage may be converted into translucent growth within another 6 to 7 hours incubation, by the consolidation of the adjacent zones (see fig. 3).

The secondary cultures isolated from within the zone do not regain the ability to produce mucoid colonies upon repeated inoculation into broth. Their biochemical activities parallel those of the parent mucoid strains, but in some instances certain changes have been observed. The secondary culture produced through the action of phage B on strain EGS became Methyl-red negative, whereas the smooth culture is Methyl-red positive, and the secondary culture produced by the action of phage B11/24 on strain 15 is Voges-Proskauer negative, while the smooth strain is Voges-Proskauer positive. Randall (1939) has also reported that certain of his variants produced by growing *Klebsiella* strains in lithium-chloride peptone water had biochemical reactions different from the original strains.

Pure line bacteriophages producing plaques with accompanying zones were isolated from some of the original sewage filtrates, using culture Egs as host strain. These pure-line phages produced plaques which did not differ from those observed when the original filtrates were used, the zones having the same ability to diffuse throughout the culture, so long as it remained mucoid. When the cultures were held at room temperature after the

plaques had appeared, while the zones diffused a little more slowly than at incubator temperature they ultimately spread through the mucoid culture until only translucent material remained. If only a few plaques were present originally, this process at room temperature took as long as three weeks—long after a culture is considered to be susceptible. Again, as with *Aerobacter* phages acting on secondary, non-capsulated strains, phages acting on *Klebsiella* Type B secondary cultures produce plaques but the plaques do not have any accompanying zones.

PHAGES ACTING ON KLEBSIELLA PNEUMONIAE TYPE A STRAINS

Four strains of Type A were employed. Two of these, Sc., and F5 were from Dr. Julianelle's collection, while the other two, Hlp.

TABLE 3

Action of sewage filtrates isolated against Klebsiella pneumoniae Type A strains

Klebsiella pneumoniae

PHAGE	TYPE A				TYPE B			TYPE C F10	KL. RHNO-SCL.	KL. OZENA.	KL. GRNU-LOMTIS.
	Sc	F5	Hlp.	Mag.	Egs	2328	17				
A Sc/1	4+	4+	4+	1+	0	0	0	0	2+	0	0
Eg Sc.......	4+	4+	1+	1+	0	0	0	0	0	0	0
Eg AF5/1 .	4+	4+	4+	4+	0	0	0	0	1+	0	0
Eg AF5/2	4+	4+	0	2+	0	3+	3+	0	4+	2+	0
Eg AF5/3	4+	4+	0	4+	0	0	2+	0	1+	3+	0
Eg AMag.. ..	4+	4+	0	4+	0	0	2+	0	4+	2+	0

and Mag. were isolated in this laboratory. All four strains agglutinated with Type A anti-serum; were pathogenic for white mice; and produced large capsules on Savita medium. None of these strains produced indole. Six phages active against these strains were isolated from sewage—two of these using culture Sc. as the host strain; three using culture F5 as host and one using culture Mag. as the strain added to the sewage broth mixture. The activity of these sewage filtrates against the various cultures is indicated in table 3.

While all of the primary sewage filtrates lysed the Type A strains certain of them also acted positively on two Type B strains. In sharp contrast to all of our other sewage filtrates three of the six produced against Type A strains as host also had lytic

power against a strain of *Klebsiella ozenae*.   All of these filtrates also lysed the single strain of *K. rhinoscleromatis* that we tested. On passing any of these filtrates against their homologous culture the ability to lyse strains other than Type A, and *K. rhinoscleromatis* was lost.   In other words, the filtrate became type specific.

These filtrates when properly seeded on a plate previously layered with any of the smooth, mucoid Type A strains, after an incubation of 12 hours exhibited plaques having large zones (see figs. 1 and 2).   The spread of these zones on increased incubation, either at room temperature or that of the incubator, duplicated in every detail the description of the spread of zones surrounding plaques of Type B phages.

Secondary cultures within the zones, or produced in broth at the expense of one of these phages, did not possess capsules, and were in most instances avirulent for white mice when injected intra-peritoneally.   However, heart-blood cultures from mice that did die after being injected with secondary culture did not produce mucoid colonies on plates, and were not susceptible to the phage that was responsible for their growth.   At intervals of 10 to 16 days, mice injected with secondary cultures produced from pathogenic Type A strains and which showed no ill effects, were sacrificed and their entire spleens removed aseptically.   The spleen was placed in a tube of broth and incubated for at least five days before being discarded as negative.   Growth when it was observed appeared by the third day, and this growth when studied was found to consist of the secondary culture to which the animal was originally subjected.

PHAGES ACTIVE AGAINST KLEBSIELLA PNEUMONIAE TYPE C

We have only two strains of *Klebsiella pneumoniae* Type C, and utilizing culture F10 (from Dr. Julianelle's collection) as host strain we have isolated three phages from sewage filtrates.   The activity of these filtrates on the Type C strains and certain other cultures is summarized in table 4.

These filtrates lost their ability to lyse *Aerobacter aerogenes* strain #11 when they were passed serially three times against either of the two Type C cultures.   However, they retain their

activity to lyse the strain of *K. rhinoscleromatis*.   These phages also produce plaques with surrounding zones when plated against smooth, mucoid, homologous culture.   The spread of these zones parallels in all respects that observed with the other phages previously described.

We have also isolated four mucoid strains of coliform bacilli from individuals with cystitis and colitis.   All four strains produced indole and were Methyl-red positive, and one strain in addition was Voges-Proskauer positive.   Phages isolated from sewage against these four strains were strain specific, lysing only the particular strain of *E. coli* that had been added to the sewage-water-broth mixture prior to incubation.   These phages when

TABLE 4

Action of sewage filtrates isolated against Klebsiella pneumoniae Type C F10

Klebsiella pneumoniae

PHAGE	TYPE C		TYPE A 8C	TYPE B 8G8	AER. 8#3	AER. #11	KL. RHNOSCL.
	F10	Thck.					
Eg C/1	4+	4+	0	0	0	3+	3+
Eg C/2	4+	4+	0	0	0	3+	3+
Eg C/3	4+	4+	0	0	1+	3+	3+

placed against their homologous cultures produced plaques with zones which on continued incubation diffuse through the mucoid growth in the same manner as we have noted previously for other phages acting on mucoid strains.

SUSCEPTIBILITY OF SECONDARY CULTURES

The phages that were utilized for the production of secondary cultures from smooth, mucoid susceptible strains were either type or strain specific.   A phage was considered to be a type-specific one when it lysed only cultures belonging in that particular group, as for example a phage that lysed only *Klebsiella pneumoniae* Type A strains.   As described in an earlier section, this high degree of specificity could be obtained when any of the sewage filtrates was passed serially against a susceptible member of that type.   Certain of the phages on first isolation were strain specific,

particularly those acting on mucoid varieties of *E. coli*, the one active against *Aerobacter aerogenes* strain AW1, and the one lysing *Klebsiella granulomatis*. For the production of secondary cultures the following method was used: To each tube of Savita broth (10 ml.) was added one drop (0.05 ml.) of a fresh 18-hour broth culture of the smooth strain and five drops (0.25 ml.) of phage. The tubes were kept in the incubator until growth appeared. In general complete clearing in the tube containing phage and culture lasted approximately 48 hours and then growth appeared. With certain of our Type B phages and those active against *Aerobacter aerogenes* S #3 frequently two weeks elapsed before any secondary growth was noted. On plating these cultures non-mucoid, translucent colonies appeared. Capsules could not be demonstrated by an India ink method. When these

TABLE 5

Action of phages on smooth and secondary culture of Klebsiella pneumoniae Type B

	BP. B11/24	BP. AF5/1	BP. C/1	BP. AER. S3
Secondary culture EGS.	0	2+	2+	3+
Original culture EGS ...	4+	0	0	0

secondary cultures were tested for susceptibility to phages other than those that produced them we noted that, in contrast to the high degree of phage specificity exhibited by the smooth culture, most of the secondaries were susceptible to phages that had no effect on the smooth parent strain. An example of the marked difference in susceptibility between a secondary culture of Kl Type B and the original mucoid strain is set forth in table 5.

The secondary cultures of Type A were not found to be susceptible to phages to which smooth parent strains were resistant, but this class of secondary cultures was the exception, most of the other secondaries that we produced giving results similar to those above.

DISCUSSION

Bacterial strains which under ordinary conditions of growth produce mucoid colonies and which when viewed under the mi-

croscope are encapsulated, are as susceptible to bacteriophagy as organisms that are commonly regarded as non-mucoid, or non-capsulated. The phages that attack such strains are usually found in sources that are rich in lytic principle for all of the members of the enteric group of bacteria. When placed in proper dilution on plate cultures of smooth, mucoid varieties of bacteria belonging to the genera *Aerobacter*, *Klebsiella*, and *Escherichia*, these phages produce plaques varying in size, and surrounding the majority of these plaques are zones, which in most instances are considerably larger in size than the plaques.

A considerable number of investigators have studied phages from the standpoint of plaque morphology and have observed plaques which have accompanying zones. We have especially observed this when studying sewage filtrates for their ability to produce plaques on cultures of *E. coli*. Such zones, however, do not increase in size on further incubation over a period of several days. Furthermore, when plaques accompanied by zones were picked for further purification with the idea of eventually making a pure line phage, it was observed that with each passage and plating the zone diminished in size and that often by the third serial passage the plaques that appeared did not possess zones.

In 1929 Sertic described a bacteriophage that acting upon a strain of *E. coli* produced plaques with zones, and that with increased incubation time the zones spread throughout the culture. The material in the zone was found to contain a lytic principle, but this was not transmissable in series; and acted on dead as well as living culture. This material which Sertic designated as the *lysin* of the phage will pass through ultra-filters that will retain the phage. This very interesting report of Sertic has been corroborated by Asheshov (personal communication) and Lominski (1937, 1939). More recently Sertic (1935) has described other phages that produce plaques with surrounding zones, and from which a lysin may be demonstrated. Dr. Sertic has very kindly sent us his susceptible strain of *E. coli* (Fb) with a pure line phage (FCZ) and we have in the main been able to repeat his experiments. However, on numerous occasions when we studied other phages isolated by ourselves which produced plaques with

surrounding zones on strains of *E. coli,* on attempting to isolate a lysin from these zones, we failed.   In those instances only phage, and not lysin was obtained.

The zones surrounding plaques produced by phages that we isolated for mucoid strains of bacteria will not disappear when these phages are passed serially.   So long as the cultures remain in the "S" phase the phages always reproduce plaques with zones. The plaques have never been observed to increase in area after the first twenty-four hours, regardless of the length of incubation period.   In distinct contrast to this, the zone surrounding the plaque regularly increases in size so long as there remains mucoid growth on the plate.   The course of the zone is easily followed since there is a difference in the appearance of the culture within the zone.   Here the culture is vitreous looking, whereas outside of the zone culture, that has as yet remained untouched, it is mucoid and opaque.   So long as any mucoid growth remains on the plate the zone continues to spread until the culture has lost completely its mucinous character.   It is important to keep in mind that a few weeks may elapse before the entire plate culture has been acted upon by the phage, the period of time depending on initial number of plaques and the incubation temperature.   With a single plaque it may take as long as three weeks at incubator temperature; with many plaques the zones coalesce and the process of decapsulation may be only a matter of several hours after plaques have appeared.

Some of these phages produce plaques that are free from secondary colonies and from within the plaque phage may be re-isolated, passed in series against homologous culture and the final filtrate may again give rise to plaques when plated.   From the zone, however, living culture can always be isolated.   This culture is free from capsule, does not produce mucoid colonies, and always carries bacteriophage.   That this is phage and not lysin is evidenced by showing that filtrates of these cultures produce plaques; that the filtrate can be passed in series against susceptible mucoid strains; and that the filtrate is inactive against the vitreous culture within the zone.   In a later report experimental evidence in detail, dealing with our attempts to prove that the zone surround-

ing a plaque is something other than phage, will be given.   When one attempts to demonstrate plaques by placing phage (from broth) on a twenty-four-hour old culture of a mucoid strain it generally results in failure.   And yet in cultures that are weeks old, the phage manifests itself by the increase in the spread of the zone through the mucoid growth.

The phage obtained from the zone will lyse only smooth mucoid culture, but not the culture from the zone (which is its secondary). It is very remarkable, however, that the phage obtained from the plaque lyses (on cross tests) not only the smooth mucoid culture, but the secondary from the zone as well.

At first glance it would appear that we were therefore dealing with two different phages; only one being present in the zone, but both being present in the plaque.   Yet every attempt to separate two phages from the plaque itself has failed.   The usual replating procedures for separating pure line types failed; every plaque obtained showed both types of phage action.   Attempts to isolate a component that lyses the secondary from a component that does not, by propagating the phage against secondary cultures, have also failed; after many such serial passages, the resulting phage produces plaques that have the same phage-composition as the original plaques (or, are identical in every way with the original plaques).

We are forced to conclude, therefore, that if we are actually dealing with two phages, then there is one in the plaque, which lyses both cultures; and that the one in the zone is continually derived from this parent phage by some process analogous to antigenic phase variation in certain bacteria.

Plaques are regularly produced by phage against secondary cultures of these mucoid strains, but no zones characterize these plaques.

The reason for the continued spread of the phage on mucoid cultures is not known.   The change brought about by the action of the phage is not, as far as we can observe, lysis of the cell body, but only a decapsulation, a "melting away" of the mucinous substance; and what remains is living secondary culture.   This may be because of the relatively low degree of susceptibility of plate cultures many days old, resulting only in an incomplete action on

the part of the phage. Or the possibility exists that even on a plate culture many days old some cell proliferation is going on and the phage multiplying against this susceptible portion of the culture gives the appearance of diffusion. However, one can demonstrate the high degree of diffusibility of these phages by seeding plates in such a manner with susceptible culture that between the
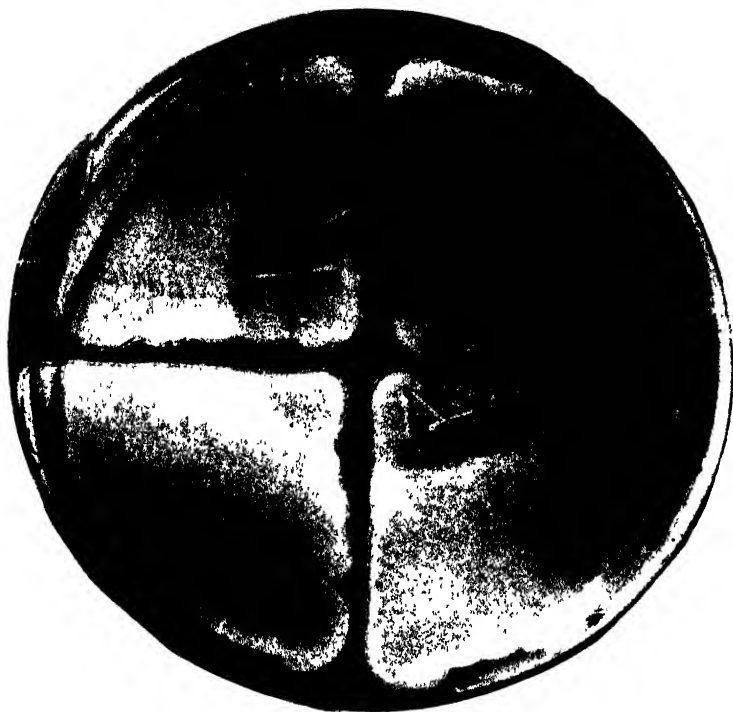
Fig. 4. Bacteriophage B Acting on Type BEgs.
Eight days incubation at 35°C. Note the spread of the phage (Zone) across bare uninoculated agar.

areas of culture considerable uninoculated agar separates them. Touching only one of the inoculated culture areas with phage, one may observe after several days of incubation the zone surrounding the original plaque has spread across the uninoculated bare agar and coming in contact with mucoid culture has decapsulated it and continues to do so as long as mucoid growth exists (fig. 4). If one cuts out of the plate a portion of the bare unino-

culated agar in the path of the diffusing phage, and places it in a
tube of broth with susceptible culture bacteriophagy occurs.
Finally the rate of spread on a plate after two weeks at room
temperature is just about the same as that which one observes
during the first three days. If the increase in the zone is due to
the presence of some enzyme system in the phage, acting partic-
ularly on the capsular substance of the organism we have not
been able to demonstrate it.

That the nature of the bacterial capsule does play some role in
a culture's susceptibility to bacteriophage is evidenced by the
type-specific action of phages attacking members of the *Klebsiella
pneumoniae* groups.   So long as these strains remain encapsulated
they are susceptible to phages active against only that particular
type.   Without capsule they become susceptible to phages acting
on other types.   Type A strains may however, prove to be an
exception to this statement, secondary cultures of Type A strains
that we have worked with being susceptible only to phages that
act on the smooth strains in this group.   Providing one has a
specific phage acting on a single type of *Klebsiella pneumoniae*
this phage may be useful in classifying organisms belonging in this
group.

CONCLUSIONS

The findings that have been described in this report may be
summarized thus:

1. Bacteriophages acting on mucoid strains of bacteria are
type-specific when acting on members of the *Klebsiella* group of
organisms, strain specific for mucoid varieties of *Escherichia*, and
may be strain or fairly group specific for organisms of the genus
Aerobacter.

2. These phages have an unusually high degree of diffusibility,
spreading through plate cultures long after the organisms have
reached their most active period of proliferation.

3. As a result of this action, the mucoid culture loses its capsule,
becomes susceptible to phages which may not act on the mucoid
phase, and is no longer pathogenic for white mice.

4. The conversion of a smooth encapsulated pathogenic species

to what may be termed on "R" state may be brought about within a short period of time by the action of a bacteriophage.

5. Specific phages may be useful in classifying *Klebsiella* strains into their respective types.

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RELATION BETWEEN FOOD CONCENTRATION AND SURFACE FOR BACTERIAL GROWTH[1]

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Storage of waters, underground, surface, sea and sewage, removed from their natural environment results in an initial multiplication of bacteria. The initial increase is reflected in the total plate counts as well as in the numbers of coliform organisms. The increase cannot be attributed to the change in temperature, as incubation of the waters at the same temperatures as in their natural environment does not prevent it. This phenomenon is the more puzzling because the waters in their natural environment do not undergo similar increases.

Zobell and Anderson (1936) obtained greater increases of bacteria in sea water stored in small volumes. This was attributed to the contact of the water with the proportionate larger solid surface area in small receptacles. The solid surfaces provide a resting place for periphytes as well as concentrating the nutrients in a film thereupon. The bacteria may multiply upon the solid surface without necessarily being firmly attached to it.

Stark, Stadler and McCoy (1938) found that in support of Zobell's results, measurable amounts of organic matter accumulated over a period of hours on the surface of chemically clean glass slides suspended in sterile lake water, indicating that the accumulation of organic matter is independent of and precedes bacterial growth. Butterfield (1933) on the other hand, found that size of container does not make any difference in the increase

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of bacteria in stored river water. That volume-surface ratio does not influence bacterial growth curves obtained in culture media was indicated by Rahn (1932), but all the waters which manifest an initial increase in bacteria are poor in nutritive value for bacterial growth. Even sewage is poor in nutrients in relation to culture media. Zobell and Anderson (1936) state that the effect of surface area on bacterial multiplication is manifest only in dilute solutions. When 100 mgm. of peptone and 1 mgm. of KNO₂ were added per liter of sea water, there was no difference between small and large receptacles in the rate of nitrite reduction.

There is little information available in regard to limiting concentration of food materials on the growth of different bacteria. Butterfield (1929) found that 0.5 mgm. each of glucose and peptone per liter, though not optimum for the growth of *Aerobacter aerogenes* was by no means a limiting concentration as the organisms multiplied after an extended lag period. Friedlein (1928) reported that *Escherichia coli* did not grow in a sodium lactate medium at concentrations lower than 0.1 per cent (1000 p.p.m.) and in a glucose medium in concentrations below 0.016 per cent (160 p.p.m.). Bacterial growth was determined by turbidity measurements and it is a question whether the failure to obtain increases in turbidity at these low food concentrations can be taken as a positive proof of the absence of growth.

Experiments reported below were therefore undertaken to secure the following information:

(1) The limiting concentration of nutrients for the growth of *Escherichia coli.*

(2) The effect of increasing the solids surface on the limiting concentration of food for the growth of *Escherchia coli.*

(3) The concentration of food materials at which the addition of surfaces fails to exert an influence on the growth of *Escherichia coli.*

(4) Comparison of the type of surface on the growth of natural water flora.

(5) Effect of biologically active surface on the growth of bacteria in water.

METHODS

Glucose and peptone were selected as the nutrients. A series of concentrations containing from 100 p.p.m. to 0.5 p.p.m. of glucose and of peptone were made. The menstruum was 50 p.p.m. phosphate buffer water. Twenty milliliter portions of the different concentrations of the media were placed in 250 ml. Erlenmeyer flasks. To one series of flasks 50 grams of 4 mm. glass beads were added in conjunction with the medium while the other series served as control without glass beads. The glass beads, as well as all glassware, were thoroughly treated with cleaning solution and washed. A sufficient number of flasks of each concentration of the medium was made so that after sampling, the flask was discarded. The medium was sterilized and inoculated with a suspension of a 24-hour culture of *Escherichia coli*. The organism was harvested from agar slopes, washed free of nutrients by centrifuging two times, and suspended in phosphate buffer. One milliliter portions of the inoculum were added to each flask and incubated at 20°C. At intervals, a set of flasks representing different concentrations of nutrients with and without the beads was shaken vigorously for 1 minute and the number of organisms determined by agar plates incubated at 37°C. for 48 hours.

RESULTS

A set of typical results is given in figure 1. The parallel determinations from duplicate flasks showed a satisfactory agreement. The results show that at 0.5 p.p.m. glucose and peptone concentration the *Escherichia coli* failed to grow in 72 hours in the absence of beads. With 2.5 p.p.m. food concentration the growth was only slight. In the presence of beads on the other hand there was considerable growth even at 0.5 p.p.m. glucose and peptone concentration. The growth level attained with glass beads between 0.5 and 12.5 p.p.m. concentration was practically the same. The numbers of *Escherichia coli* were higher in the presence of beads over the corresponding concentrations without beads up to 25 p.p.m. Beyond this concentra-

tion the numbers of organisms were practically the same whether beads were present or not.

Although *Escherichia coli* can grow both under aerobic and anaerobic conditions, the objection might be raised that the ob-
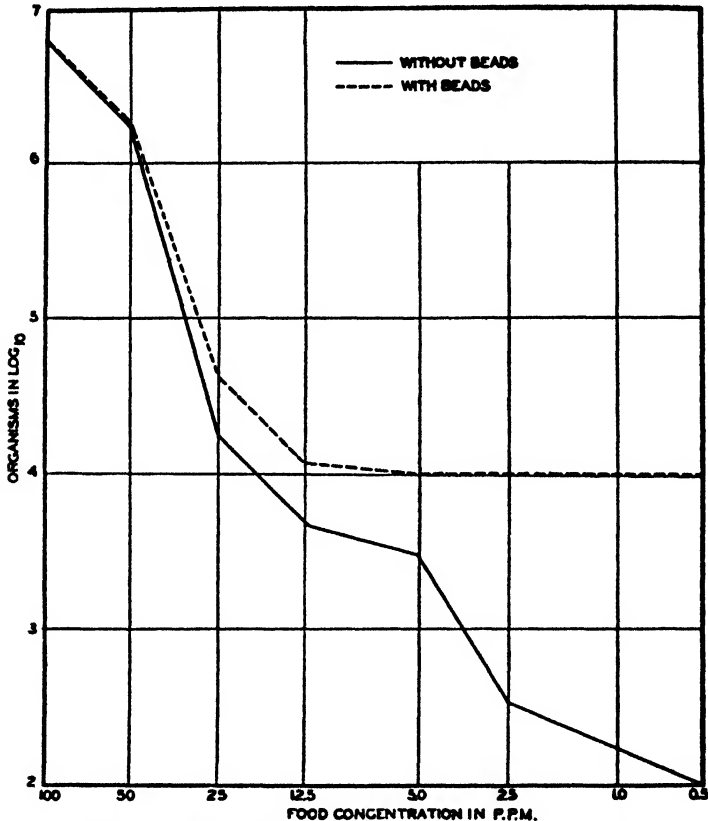
FIG. 1. NUMBERS OF ESCHERICHIA COLI (IN LOG₁₀ PER ML.) IN VARIOUS CONCENTRATIONS OF GLUCOSE AND PEPTONE DURING 72 HOURS' INCUBATION WITH AND WITHOUT GLASS BEADS
(Initial number 170 per ml.)

served results were affected in the higher concentrations by the absence of dissolved oxygen. That this was not the case is indicated from the results of the following experiment. The same medium used previously was distributed into 1 liter flasks in 300 ml. quantities. The concentrations of glucose and peptone

varied from 0.5 p.p.m. to 100 p.p.m.   The media were inoculated with a 24-hour culture of *Escherichia coli* and incubated for a period of 48 hours.   At the end of this period the medium was siphoned carefully into 150 ml. glass stoppered bottles and dissolved oxygen determined according to the Rideal-Stewart modification of the Winkler method.   The results are given in table 1. Dissolved oxygen was present in all the concentrations of glucose and peptone except in the 100 p.p.m.   In these large flasks there was a surface-volume ratio of 15:1, whereas the surface-volume ratio in the 250 ml. flasks with 20 ml. portions the medium

TABLE 1

*Dissolved oxygen content of different concentrations of glucose peptone broth inoculated with Escherichia coli**

CONCENTRATIONS	DISSOLVED OXYGEN
p.p.m.	*p.p.m.*
100.0	0
50.0	4.0
25.0	7.8
12.5	8.0
5.0	8.0
2.5	8.0
0.5	8.0

* After 48-hour incubation of 300 ml. portions in 1 liter Erlenmeyer flasks.

was 2:1.   It is therefore reasonable to assume that dissolved oxygen was present in the smaller flasks containing 20 ml. of the medium even at 100 p.p.m. of glucose and peptone.   Hence the results reported in the first experiment were not influenced by the differences in dissolved oxygen content at various food concentrations.

The effect of different types of surface on bacterial numbers was then compared.   The menstruum was unpolluted surface water with its natural population.   The water was placed in beakers and (1) kept under quiescent conditions, (2) aerated, (3) agitated continuously by means of paddles, and (4) kept in contact with sand.   Thoroughly cleaned and washed fine Ottawa sand was used.   The volume of water added was just sufficient

to wet the sand. In order to obtain representative samples known quantities of sterile water were added to the sand, and vigorously shaken, after which the sand was discarded. Bacterial numbers were determined by nutrient agar plates incubated at 20°C. for 48 hours. The results are given in figure 2. The bacteria increased in the control up to 24 hours and thereafter
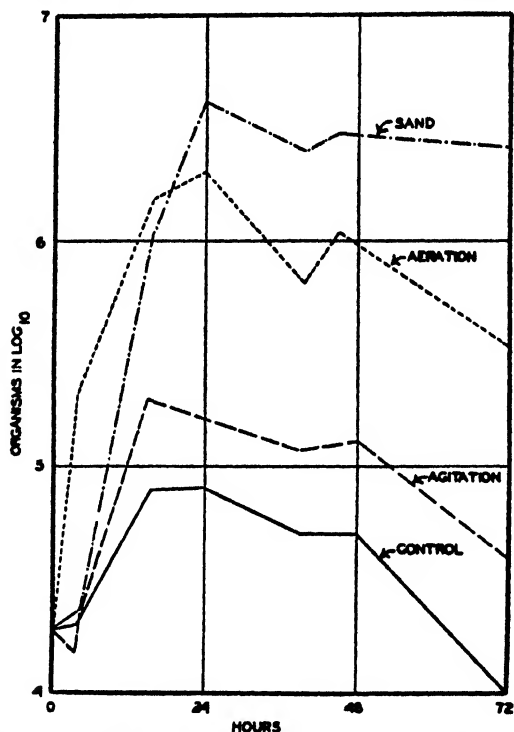
FIG. 2. NUMBERS OF BACTERIA (AGAR PLATE COUNTS) IN SURFACE WATER (a) KEPT UNDER QUIESCENT CONDITIONS, (b) IN THE PRESENCE OF SAND, (c) AGITATED, AND (d) AERATED

decreased gradually up to 72 hours when there were fewer organisms remaining than initially. Agitation caused an increase of organisms to a higher level than were present in the control. With aeration and with sand the numbers were increased to even higher levels. The numbers dropped after 72 hours aeration but the number of organisms remaining at this time was greater than the initial number. With sand, the numbers did not show an

appreciable decrease even after 72 hours.   The aerated water did not show an initial lag period.

The effect of gentle stirring of sewage by means of submerged paddles on bacterial numbers is illustrated in table 2.   It is unlikely that reaeration was materially increased by the stirring to influence the results.   The numbers in the unstirred sewage did not increase while in the stirred sample there was a 2.5-fold increase.

TABLE 2

Effect of stirring of sewage on bacterial numbers

Bacteria in millions per ml.

TIME	NOT STIRRED	STIRRED
hours		
0	8.2	8.2
4	8.0	7.4
24	4.3	21.2

TABLE 3

Effect of addition of stones covered with biologically active slime on the numbers of bacteria in water

Bacteria in thousands per ml.

TIME	NON-POLLUTED WATER		POLLUTED WATER	
	Control	Stones added	Control	Stones added
hours				
0	22	30	316	303
9	30	27	1,100	795
26	800	230	6,850	3,900
72	230	23	416	37
96	37	110	30	64

The effect of solid surfaces covered with biological slime on bacterial numbers of the supernatant water was investigated. Stones from the bed of an unpolluted stream were collected and placed in battery jars one layer deep.   The jars were then filled with polluted and unpolluted stream water.   Controls consisted of the same waters without the stones.   The results are given in table 3.   Storage of both the polluted and unpolluted water resulted in increases in bacterial numbers, those in the polluted

water being higher. When these waters were stored in the presence of slime-covered stones, the increases in bacterial numbers were not as great.

Under the experimental conditions the limiting concentration of glucose and peptone for the growth of *Escherichia coli* was about 2.5 p.p.m. of each of the ingredients. This result was obtained by inoculating with very small quantities of thoroughly washed cells. It is conceivable that with larger inoculations the limiting concentration might be even lower. The limiting food concentration is not a fixed value but is dependent on the amount of surface in contact with the medium. By increasing the amount of surface per unit volume of the medium, as was done experimentally by the introduction of clean glass beads, the limiting concentration is moved downward and appreciable growth is obtained even at 0.5 p.p.m. concentration. Up to 25 p.p.m. glucose and peptone concentration, increasing the surface-volume ratio by the introduction of the glass beads resulted in increase in the growth of *Escherichia coli*. Beyond this value the addition of glass beads had little effect on the numbers. Furthermore, increasing the concentration of glucose and peptone from 0.5 to 25 p.p.m. had little influence on the ultimate numbers of *Escherichia coli* in the presence of glass beads. Increasing the concentration of food above 25 p.p.m. resulted in higher bacterial numbers, both with and without glass beads. It appears that food concentration was the limiting factor up to 25 p.p.m. glucose and peptone and that within this range the addition of surface enabled the organisms to produce greater growth presumably due to the surface concentration of the food and the bacteria (Stark, Stadler and McCoy, 1938; Zobell and Anderson, 1936). At 50 and 100 p.p.m. the addition of surfaces did not result in greater increases in bacterial numbers.

Escherichia coli is not a true periphyte. Therefore, the concentration of these organisms on solid surfaces is induced only by the dearth of food. Furthermore, the attachment is superficial, because shaking readily detaches them. A 24-hour growth of

this organism in the presence of glass beads, after shaking for 1 minute, yielded 41 million organisms per milliliter.   When the menstruum was poured off and the glass beads were shaken with fresh sterile water (9 ml.) an additional 2 million bacteria were obtained per milliliter.   On repeating the process a second and third time 520,000 and 500,000 organisms per milliliter were obtained, respectively.   This would indicate that the attachment of the organisms on the surface is superficial and that under quiescent conditions there may be a continuous interchange of organisms between the menstruum and the surface.   Therefore, the numbers obtained in the presence of glass beads do not represent the total numbers of organisms present but only those that could be detached by shaking.

It should also be pointed out that the minimum food concentration for bacterial growth will vary with the type of organisms. In general, the larger the organism the higher will be the limiting food concentration (Butterfield, 1929).

It is our contention that the increase in the bacterial numbers on aeration and agitation is to be attributed to surface phenomenon similar to those which govern the action of sand or glass beads.   That the increase is not due to dispersion of the organisms originally present in the water is indicated by the fact that agitation for the first five hours did not result in an increase. Dissolved oxygen, as such, did not exert an influence on the results because the water, being unpolluted, had a low oxygen-consuming capacity and was thoroughly reaerated prior to use. The dissolved oxygen value at the end of the period was 7.4 p.p.m. in the control, 8.0 p.p.m. in the aerated sample and 7.8 p.p.m. in the agitated sample.   It is improbable that the changes in the CO_2 tension brought about by the treatment could have affected the bacterial numbers.   The differences in the bacterial numbers attained seem to be in the order of the increases in the surface. The lowest increase over the control was obtained with agitation and the highest with sand and aeration.

Sand filtration of liquid wastes such as sewage and water to obtain highly purified effluents is an old established practice. Bacteriologically this method seems to be ideally suited for the

removal and oxidation of soluble impurities from water in very low concentrations. With such low concentrations of organic material as are present in water and sewage, the only possible rapid biological action seems to be in the presence of solid surfaces enabling the surface concentration of food and bacteria. These solid surfaces soon become coated with a highly specialized slime wherein biological action takes place at a high rate. Surfaces free from slime make possible the oxidation of low concentrations of organic matter but when these surfaces become coated with slime the rate of oxidation is greatly accelerated.

A similar phenomenon is encountered in the activated sludge process of sewage treatment. In this process bacteria capable of growing in colonial form (*Zooglea ramigera*) are the predominant forms (Butterfield, 1935). The surfaces to which these organisms attach themselves may be those of inert inorganic or organic particles. The organisms grow in masses embedded in capsular gel and form large flocs. Sewage, compared with culture media represents a very dilute substrate, and contains both dissolved and dispersed material. The dispersed matter is concentrated on the surfaces of the floc and acted upon at a very high rate by the mass of organisms present in the floc. There is, however, no evidence of surface concentration of the dissolved materials, which are probably directly assimilated by the organisms during the circulation of the floc through the liquid. Mixing is therefore essential, in addition to an adequate supply of oxygen to maintain aerobic conditions. Both mixing and maintenance of aerobic conditions may be accomplished either by mechanical agitation or by aeration. Aeration and agitation have been shown to increase the number of bacteria in surface waters by virtue of increasing the internal surface of the liquid and consequent contact between the organisms and their food material.

The increase of bacteria in water removed from its natural environment and stored in the laboratory has been attributed to increase in the surface-volume relationship (Stark, Stadler and McCoy, 1938). However, when a surface water is removed from its natural environment, there is brought about in addition, a change in the relationship between the water and the stream bed.

The stream bed is covered with active biological slime which may remove bacteria from the water and prevent an increase in their numbers. On the other hand, in waters stored in the laboratory this factor is not present and the increased surface results in greater numbers. The addition of stones covered with slime to water reduced the increase in bacterial numbers over the control without the stones. The amount of active surface film in relation to the volume of water was greatly increased under laboratory conditions as compared with natural conditions. At the same time, however, the amount of food material introduced by the slime was also increased. It is probable that considerable diffusion of soluble, and some dispersion of insoluble, particles took place when the stones covered with film were placed in the water, resulting in an increase in the numbers of bacteria, while the active surfaces would tend to decrease the numbers of bacteria in water by adsorption. The net effect of the addition of slime covered stones therefore is the resultant of these two factors, an increase in the numbers but of a lower magnitude than without the slime-covered stones. A differentiation should be made between the action of inert surfaces as such, which would tend in highly dilute substrates to increase the numbers of bacteria, and active surfaces of slime which would exert an opposite effect. Under natural conditions inert surfaces are seldom encountered without an active slime.

SUMMARY AND CONCLUSIONS

For the purpose of determining the effect of food concentration and surface on the growth of bacteria, washed cultures of *Escherichia coli* were inoculated into glucose and peptone medium of concentrations varying from 100 to 0.5 p.p.m. in flasks of 250 ml. capacity containing 20 ml. of the medium. To one series 50 grams of 4 mm. clean glass beads were added. The numbers of organisms were counted by the plate method after different periods of incubation.

Under the conditions of the experiment the growth of *Escherichia coli* did not take place in glucose and peptone concentrations of 0.5 and 2.5 p.p.m. The addition of glass beads to the medium

at these concentrations permitted a considerable growth of these organisms. The effect of glass beads was noticeable up to 25 p.p.m. concentration of glucose and peptone. Beyond this concentration the numbers of *Escherichia coli* with and without beads were practically the same.

The population of stream water is similarly affected by the addition of clean sand. Aeration and agitation also result in higher counts of the flora of the water. Stirring of sewage likewise results in an increase of bacterial numbers.

The addition of stones covered with biologically active slime to surface waters does not prevent the increase in the numbers of bacteria but considerably reduces the magnitude of the increase.

Surfaces enable bacteria to develop in substrates otherwise too dilute for growth. Development takes place either as bacterial slime or colonial growth attached to the surfaces. Once a biologically active slime is established on surfaces, the rate of biological reaction is greatly accelerated.

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A LOW TEMPERATURE STORAGE CABINET FOR THE PRESERVATION OF VIRUSES

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Viruses which are unstable and become inactivated rapidly at room temperatures usually retain their activity for a longer period at lower temperatures. When maintained at temperatures below certain critical levels, even the most unstable viruses may be stored almost indefinitely with no detectable decrease in infectious titer. Equipment suitable for the indefinite storage of a large number of specimens suspected or known to contain various viruses eliminates the necessity of continuous animal passage and permits repeated and varied studies on single specimens. In the investigation of acute virus infections of the respiratory tract of human beings equipment of this kind is particularly useful since it is physically impossible to investigate simultaneously the large number of specimens which may be obtained from a given epidemic or to maintain in continuous animal passage the numerous strains of viruses which may be isolated.

Several years ago Turner (1938) described a method for the preservation of treponemata and viruses in the frozen state without loss of virulence. Specimens were placed in stoppered glass tubes and were kept in the frozen state by submersion in a mixture of solid carbon dioxide and ethyl alcohol in a vacuum-insulated container. More recently Turner and Fleming (1939) reported the results of observations on the virulence of spirochetes and filterable viruses after storage under these conditions for periods up to 3 years. Despite the reliability of this method and its advantages over other methods of preservation, there are certain difficulties in its operation. Wide fluctuations in temperature

may occur unless the proportions of the refrigerant mixture are properly maintained. Specimens may become contaminated, inactivated, or lost through the breakage or leakage through stoppers of glass tubes. Finally, individual specimens are not easily located when submerged with other tubes in the refrigerant mixture.

In order to overcome these difficulties and to provide facilities for the indefinite storage of a large number of specimens, a low temperature cabinet was designed recently in this laboratory.

FIG. 1. OBLIQUE END VIEW OF LOW TEMPERATURE STORAGE CABINET

The cabinet has proven sufficiently useful, efficient, and simple of operation to warrant this report.

The cabinet, shown in figures 1 to 3, is of double-walled construction throughout and is entirely insulated with glass-wool.[1] The outer walls and the bottom are made of galvanized iron and coated with white Duco, while the inner walls are made of stainless steel. Between the outer and inner walls is a 6-inch layer of

[1] The cabinet was constructed by Thomas Hindley and Sons, Inc., 1185 Sixth Avenue, New York, from specifications furnished by this laboratory.

glass-wool insulation. The covers of the cabinet, also of double-walled construction, contain a 4-inch layer of glass-wool insulation. Four centrally-hinged covers close the four openings in the top of the cabinet and permit access to the inner low temperature compartments. The outside dimensions of the cabinet are: length 68 inches, width 35 inches, and height 45 inches including the 4-inch supporting legs. The inside dimensions are: length 56 inches, width 23 inches, and height 26 inches.

Figure 1 shows an oblique end view of the cabinet. The two covers, A and D, close the tops of the terminal refrigerant com-

FIG. 2. TOP VIEW OF CABINET WITH FOUR COVERS REMOVED

partments, which contain solid carbon dioxide. The covers, B and C, close the top of the central storage compartment, which contains cold gas and the specimen tube racks.

Figure 2 shows a top view of the cabinet with the four covers removed. The cabinet was in actual use when this photograph and that in figure 3 were made. Each of the terminal compartments, E and F, contains 200 pounds of solid carbon dioxide. These compartments are separated from the central storage compartment of the cabinet by two heavy galvanized iron screens of ½-inch mesh. One of these screens is shown at G. Through these screens a free exchange of cold gas from the solid carbon dioxide compartments to the storage compartment constantly occurs.

The upper surfaces of the two drawer frames and part of the top of each of the twelve vertical drawers are shown at *H* and *I*. The details of the frame and drawer construction are shown in figure 4. Gas spaces $1\frac{1}{4}$ inches in width separate the drawer frames from the inner walls of the cabinet, and there are spaces of similar width between each pair of drawers. These spaces assure a free circulation of cold gas between the refrigerant and the specimen tubes. Figure 3 shows an oblique top view of the
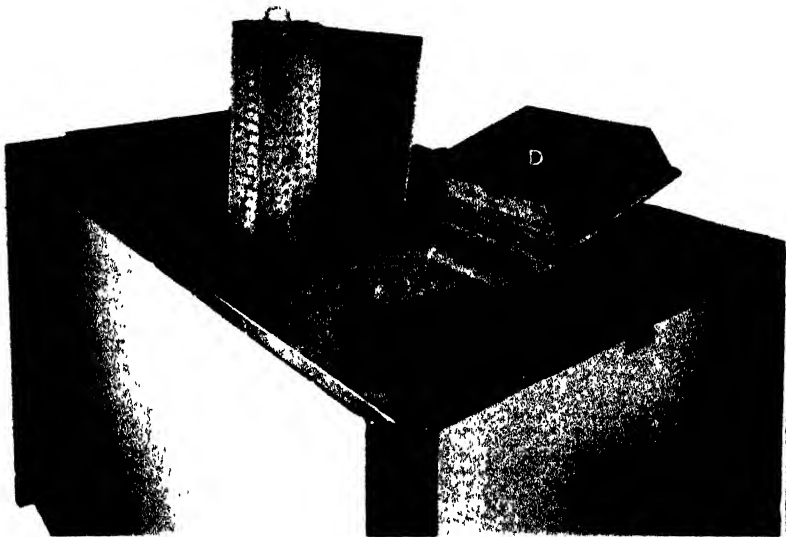
FIG. 3. OBLIQUE TOP VIEW OF CABINET WITH TWO COVERS HALF OPEN AND ONE DRAWER RAISED

cabinet with two of the four covers, *C* and *D*, half opened. The covers are hinged centrally and are provided with knob handles, as shown, in order that any compartment in the cabinet can be reached easily. Each half cover is $12\frac{1}{2}$ inches long and 14 inches wide at the top. The sides of the covers are made of wood beveled to fit the sides of the openings in the top of the cabinet. The covers contain 4 inches of glass-wool insulation. One drawer, shown at *J*, has been raised to illustrate the manner in which a specimen tube can be obtained.

Figure 4 shows an oblique view of one of the two drawer frames

which fit in the central cold gas compartment of the cabinet. The
frames are $20\frac{1}{2}$ inches long, $10\frac{1}{2}$ inches wide, and $22\frac{1}{2}$ inches high
and are made of stainless steel. Each frame contains sharp-edged
vertical slides for the support of six drawers. The drawers, as
illustrated at J, are made of perforated stainless steel sheeting and
measure 22 inches in length, $4\frac{1}{2}$ inches in width, and $5\frac{1}{2}$ inches in
depth. Each drawer is provided with a handle, shown at K, to
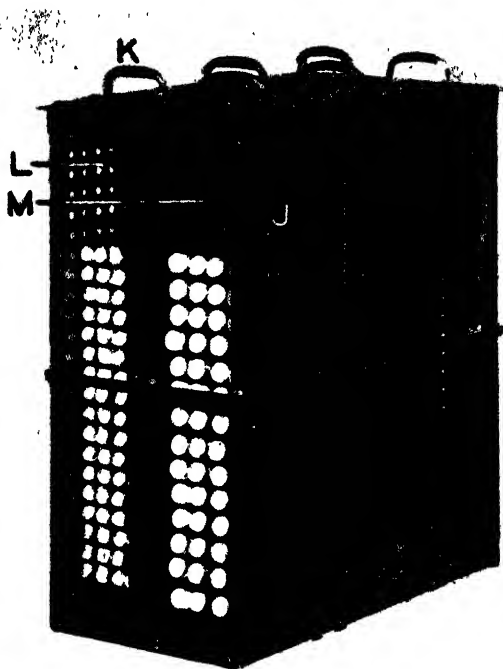
Fig. 4. Oblique Side View of One Drawer Frame and Specimen Racks

facilitate raising the drawer and its contained specimen tubes.
The drawers are fitted with metal racks, so arranged that the
specimen tubes are supported near both their upper and lower
ends and are maintained at an angle of approximately 15° from
the horizontal. In this manner any tendency of tubes to slide
forward and block the movement of the drawers is eliminated.
The racks have been made in two sizes, as shown at L and M, so
that tubes of various sizes can be used with a minimum utilization

of space. The twelve drawers have a maximum capacity of 1500 specimens in either medium- or small-sized tubes and 912 specimens in large-sized tubes.

Figure 5 shows three sizes of specimen tubes which are used in the cabinet. All of the tubes are made of celluloid and are provided with metal screw caps.[2] The large tubes, N, are $4\frac{7}{8}$ inches

Fig. 5. Three Sizes of Celluloid Specimen Tubes with Metal Screw Caps

long and $\frac{7}{8}$ of an inch in diameter, and they have a capacity of 38 ml. The medium tubes, O, are $4\frac{1}{4}$ inches long and $\frac{5}{8}$ of an inch in diameter, with a capacity of 17 ml. The small tubes, P, are $3\frac{3}{8}$ inches long and $\frac{3}{8}$ of an inch in diameter, and have a capacity of 7 ml. The celluloid tubes become soft and distorted when sterilized either in the autoclave or in boiling water. It has been found, however, that they can be completely sterilized without

[2] The tubes and caps were manufactured by the Lusteroid Container Co., Inc., 10 Parker Avenue West, South Orange, New Jersey.

undergoing physical alteration by ultraviolet-light irradiation. Open tubes are placed 12 inches from a powerful quartz mercury arc with the light directed into the lumina of the tubes and are irradiated for 1 hour. The metal screw caps may be sterilized either by autoclaving or by dry heat. Celluloid tubes have the advantage over glass in that they do not become brittle at the low temperatures maintained in the cabinet and do not break when solidly frozen specimens are rapidly thawed. Screw caps have been chosen in preference to rubber or cork stoppers because the former cannot be forced off the tubes by the gas expansion coincident with thawing.

FREEZING AND THAWING OF SPECIMENS

Specimens are placed in tubes of appropriate size and the metal caps are screwed in place. The tubes are then partially immersed in a mixture of solid carbon dioxide and alcohol kept for this purpose in one terminal compartment of the cabinet. The specimens are frozen solid in a few seconds, and later, at convenience, the tubes are transferred to a suitable place in one of the storage drawers. The location of the specimens is recorded in a card index. When a particular stored specimen is desired, the drawer containing it is raised sufficiently to permit the removal of the required tube and is then immediately lowered. Whenever the drawer handle is grasped, heavy leather gloves are worn in order to protect the hands from the very low temperature of the metal. Frozen specimens are rapidly thawed under running tap water or by partial immersion for a few minutes in a water bath at 37°C. Frequently only a small portion of a specimen is required, and immediately after its withdrawal the remainder of the specimen is rapidly refrozen in the manner described above and returned to the cabinet.

SOLID CARBON DIOXIDE CONSUMPTION

The two terminal compartments of the cabinet have a total capacity of 400 pounds of solid carbon dioxide. Approximately 30 pounds of solid carbon dioxide evaporate during each 24 hours, and, therefore, a similar quantity is added to that remaining in

the cabinet each day. It has been found, however, that even when no additional solid carbon dioxide is placed in the cabinet for 3 successive days, no significant increase in the temperature of the storage compartment occurs.

TEMPERATURE

During the 15 months that the cabinet has been in continuous use in this laboratory the temperature of the gas mixture in the storage compartment has been observed daily. The maximum temperature has been $-72°C.$, the minimum $-80°C.$, and the mean $-76°C.$ The storage compartment has been maintained throughout this period, therefore, at a temperature approximately $105°C.$ below that of the surrounding room air.

PRESERVATION OF VIRUSES

A considerable variety of specimens has been kept for periods up to 15 months in the cabinet. The specimens have included broth and saline throat washings, whole blood and blood serum, various animal and chick embryo tissues, tissue culture media, and a variety of suspensions of different concentrations of the tissues enumerated.

Throat washings obtained from patients during an influenza epidemic in January 1939 (Horsfall, Hahn and Rickard, 1940) and shown to have contained influenza virus were stored in the cabinet for 10 months. At the end of this period they were still capable of producing typical experimental influenza in inoculated ferrets and stimulated the production of specific neutralizing antibodies in Syrian hamsters (Taylor, 1940). It has previously been reported (Horsfall, 1939) that a suspension of the PR8 strain kept under these conditions and repeatedly titered during a period of 5 months showed no detectable decrease in the infectiousness of the suspension. This suspension has now been kept for 15 months in the cabinet, and its infectious titer has remained unaltered. A large number of different strains of influenza virus have been stored in the cabinet. In no instance has the virus become inactivated nor has a decrease in infectious titer been demonstrated.

The pneumonia virus of mice, which has been shown to be even more unstable than influenza virus (Horsfall and Hahn, 1940), was stored in the cabinet for 9 months without undergoing a noticeable decrease in infectious titer. Canine distemper virus was also stored in the cabinet for 9 months. At the end of this time the virus in high dilution was still capable of producing severe distemper in inoculated ferrets.

PRESERVATION OF PATHOGENIC PROTOZOA

Coggeshall (1939) has reported that *Plasmodium knowlesi* and *Plasmodium inui* will remain viable for as long as 70 days when stored in this cabinet. These observations have been continued and also extended to include other plasmodia and trypanosomes. To date, his unpublished studies show that *P. knowlesi* has remained infectious for 140 days, *P. inui* for 151 days, *P. cathemerium* for 35 days, *Trypanosoma brucei* for 135 days, *T. duttoni* for 34 days, and *T. equiperdum* for 62 days. These figures represent time thus far covered by observation, and it seems highly probable that these parasites will remain viable considerably longer. On the other hand, there have been occasional specimens encountered which have lost their infectivity.

DISCUSSION

The low temperature storage cabinet described in this paper was designed to accommodate a large number of virus specimens in an easily accessible manner and to maintain them in a state of unaltered infectiousness. The preservation of the activity of unstable viruses for periods of months makes possible intermittent or delayed study of a given specimen. The systematic storage of specimens in numbered racks permits the rapid location of any particular tube. The specimen tubes are surrounded simply by a cold gas mixture resulting from the evaporation of solid carbon dioxide, and there is, therefore, no possibility that a virus-inactivating agent, as for example alcohol, might enter the tubes. The use of celluloid instead of glass tubes eliminates the loss of specimens resulting from cracked, or broken tubes, and the substitution of metal screw caps for stoppers prevents the un-

stoppering of tubes due to gas expansion during thawing. The small-sized celluloid tubes afford an additional advantage since they are of dimensions and material suitable for use in the high speed vacuum centrifuge (Bauer and Pickels, 1936) and, therefore, permit the direct concentration of active material in the original storage container.

SUMMARY

A storage cabinet of large capacity suitable for the indefinite preservation of unstable viruses has been designed. The cabinet contains solid carbon dioxide and cold gas and maintains an internal temperature of $-76°C$.

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INDIVIDUAL ISOLATION OF INFECTED ANIMALS IN A SINGLE ROOM

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Certain virus diseases are highly infectious and are transmissible by contact. In some, the mere physical proximity of normal animals to infected animals is sufficient to cause infection in the former. This degree of infectiousness is common to a number of viruses which invade the cells of surface tissues, particularly the mucous membranes of the respiratory tract and the skin. Influenza (Smith, Andrewes, and Laidlaw, 1933), psittacosis (Lazarus and Meyer, 1939), distemper (Dunkin and Laidlaw, 1926), lymphocytic choriomeningitis (Traub, 1936 and Coggeshall, 1939), infectious ectromelia (Marchal, 1930), malignant panleucopenia (Hammon and Enders, 1939), and vaccinia (Parker and Rivers, 1936) viruses, to mention but a few, are typical examples.

In the study of some virus diseases it is absolutely essential that the possibility of contact infections be eliminated. This is particularly true in the case of influenza since accidental infections, either apparent or inapparent but resulting in the development of specific antibodies, may vitiate the interpretation of results. Ferrets which are exposed, even at some distance and for relatively short periods, to other ferrets in the acute phase of influenza usually contract the disease (Smith, Andrewes, and Stuart-Harris, 1938). This fact complicates attempts to identify the virus in material obtained from human beings and makes it necessary that inoculated ferrets be strictly isolated. Furthermore, ferrets often fail to show typical symptoms following the inoculation of human throat washings which contain the virus

(Horsfall, Hahn, and Rickard, 1940). In these instances the determination of the presence of influenza virus in the inoculum is usually dependent upon the demonstration of the development of specific neutralizing antibodies by the ferret. Unless it is certain that the animal did not contract a subclinical infection accidentally, the development of antibodies loses all significance in relation to the inoculum. Finally, the significance of studies on the antigenic differences between strains (Magill and Francis, 1938) is dependent upon keeping different strains pure and free from accidental contamination by other strains of the virus. In the absence of adequate facilities for the isolation of animals infected with different strains, it is probable that cross infections occur.

AN ARRANGEMENT FOR THE ISOLATION OF INDIVIDUAL ANIMALS

The purpose of this paper is to report the technique and equipment developed in this laboratory by which it was found possible to insure strict individual isolation of a number of experimentally infected animals in a single room. The essential element of this arrangement is a series of relatively airtight metal cubicles each connected with a forced draft ventilating system. The air intake, as well as the outlet, of these cubicles is provided with filters, and each cubicle can be disconnected and removed from the ventilating system without interfering with the ventilation of the others. This isolation system has been in continuous operation for 6 months, and has proven to be reasonably simple to operate and thoroughly effective in preventing cross infection among experimental animals. It has been used for the isolation of animals ranging in size from mice to dogs and monkeys. These animals have been inoculated with various strains of influenza or distemper viruses, and in no instance has an accidental cross infection been observed. Following is a description of the details of the isolation system.

Isolation cubicles and the ventilating system. An individual isolation cubicle is shown in figure 1. It measures 24 by 24 by 20 inches, and in order to resist the effects of frequent washings and

antiseptic sprays, it is made of 22-gauge monel metal sheeting.[1] The front is closed by a tightly fitted door which is provided with a reinforced glass window measuring 13 by 17 inches. The periphery of the door is completely surrounded by a metal rim, 2 inches wide, which overlaps the walls of the cubicle. The door is supported by butt-hinges and can be removed for cleaning and sterilization. A hinged lock holds the door closed and keeps its inner surface tightly pressed against a contact flange on the walls of the cubicle. Because of this construction the cubicle is practi-

Fig. 1. Front View of Isolation Cubicle Showing Reinforced Glass Window and Air Filter Holder at Air Intake

cally airtight when closed. Air is admitted to the cubicle through an inlet vent in the door. The vent is 2 inches in diameter and contains, 1 inch from its end, an inset metal screen. The vent is covered by a tightly fitting metal cap which also contains a metal screen. The space between these two screens is provided for a suitable air filter. Air is withdrawn from the cubicle through an outlet vent placed in the middle of the back wall 1 inch from the top. The construction of the outlet vent is identical to that of

[1] Manufactured by **P.** Feiner and Sons, Inc., 552 West 52nd Street, New York.

the inlet vent except that the cap and the filter in the former instance are inside the cubicle.

Figure 2 shows the front of a cubicle with the door open and an animal cage in position within.   The overlapping rim of the door, the contact flange of the walls, the air inlet and outlet vents are shown.

The floor plan of the entire isolation unit and the plan of the ventilating system are shown in figure 3.   There are four banks of isolation cubicles, each three tiers high, accommodating a total
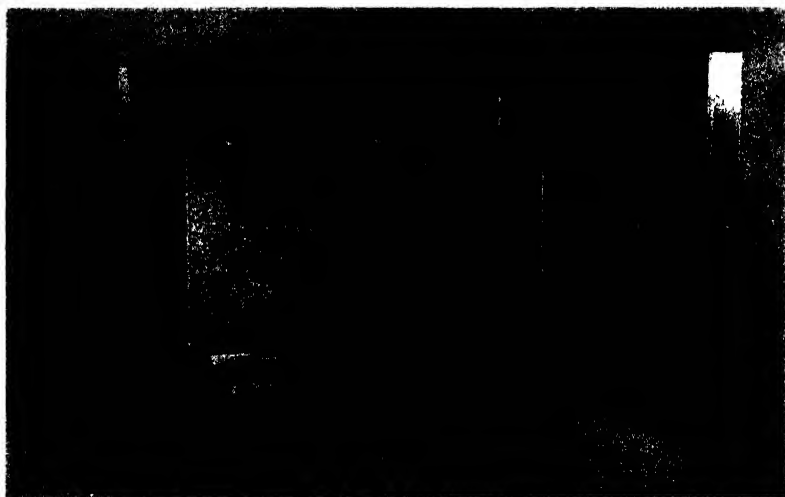
FIG. 2. ISOLATION CUBICLE WITH DOOR OPEN AND A FERRET CAGE IN POSITION
Air outlet with its filter holder is also visible

of 51 cubicles in the isolation room.   These banks of cubicles, two in the center and one at either end of the room, are supported by hanging racks suspended from steel beams and, as seen in figure 4, do not touch the floor.   The ventilating system consists of a main air duct 14 inches in diameter provided with a powerful suction fan having a capacity of 1800 cubic feet of air per minute. This duct passes over the banks of cubicles in the center of the room and then separates into two branches which extend to the two banks at the opposite ends of the room.   Arrows in figure 3 indicate the direction of air flow.   As seen in figures 4 and 5,

branching out from the main horizontal ducts are secondary vertical ducts, one for each separate column of three cubicles. The lower portion of the secondary branch ducts is rectangular in shape, and, at the level corresponding to the air outlets of each of the three cubicles, it is provided with circular openings slightly
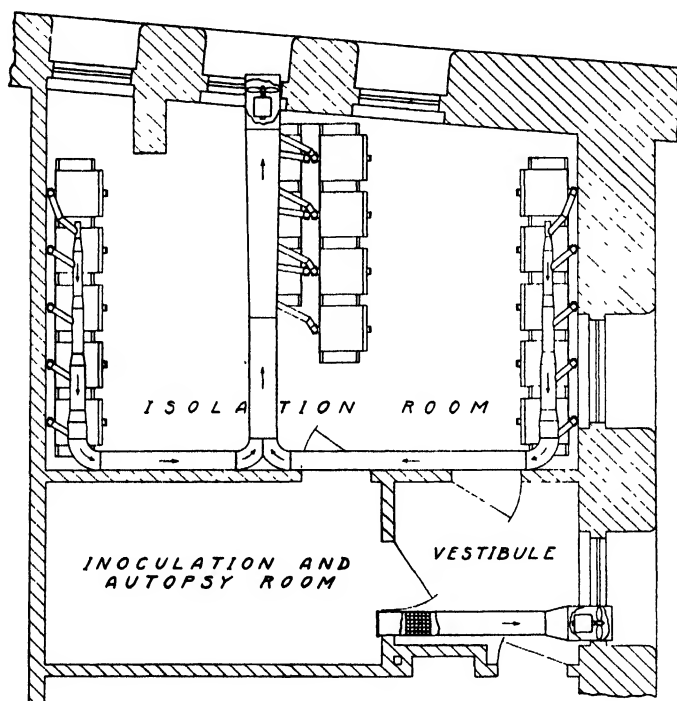
FIG. 3. FLOOR PLAN OF ISOLATION UNIT AND DIAGRAM OF VENTILATING SYSTEM

Location and distribution of cubicles in isolation room is indicated. Secondary air ducts branching off vertically from main horizontal duct are also shown diagrammatically. Location of adjoining room where animal inoculations and autopsies are done and vestibule leading to entire isolation unit with their separate ventilating system is also indicated.

over 2 inches in diameter. Each opening is provided with a sleeve that fits snugly over a similar sleeve surrounding the air outlet of the cubicle. The openings in the branch ducts are also provided with flat metal sliding gates by which the opening can be closed when desired. The hanging racks which support the cubicles are provided with guide rails for the cubicles. They

align the cubicles when placed on the rack and pushed into position so that the sleeve surrounding the air outlet of the cubicle automatically slips into the sleeve surrounding the opening in the vertical air duct and thus connects the cubicle with the ventilating system.

FIG. 4. PHOTOGRAPH OF INTERIOR OF THE ISOLATION ROOM
Two cubicles have been removed from one of the two banks in the center of the room in order to show openings in a vertical air duct for ventilation of cubicles. The exhaust end of the main ventilating duct at the window is visible.

Such an arrangement allows the withdrawal of any number of cubicles without interfering with the ventilation of others. Figure 5 shows one bank of cubicles from which three have been removed and the details of the openings in the vertical air duct with their sliding gates. The amount of air passing through a cubicle can be regulated by the sliding gate, but because of the resistance offered by the filters, it averages about 3 cubic feet per minute even when the gates are fully open.

Since the fan constantly withdraws air from the system, the pressure within the cubicles and in the air ducts is always slightly lower than that in the surrounding room. Consequently, any small air leaks which may be present continuously operate to prevent the escape into the room air of potentially infected air from the cubicles. The air within the cubicles is constantly drawn a-

FIG. 5. PHOTOGRAPH OF A BANK OF CUBICLES AT ONE END OF THE ISOLATION ROOM
    Three cubicles in the center of the hanging rack have been removed in order to show details of air intakes in a secondary vertical air duct. Sliding gates at the openings of air intakes are also visible.

way into the air ducts and discharged from the room. The air which enters the cubicles has had no possible contact with infected animals. In effect, therefore, the animal is confined in an airtight compartment, and air which enters must pass the inlet filter just as the air which leaves must pass the outlet filter. It was not supposed or planned that either the inlet or the outlet filters would actually sterilize the air flowing through them. They

were placed in the air vents merely to prevent dust and suspended droplets from entering or leaving the cubicles. This function these filters have accomplished quite satisfactorily.

In this laboratory the main air duct discharges directly to the outside. In the event that the unit were to be used for the isolation of animals infected with a disease highly infectious for human beings, it might be necessary to effect complete sterilization of the discharged air. Discharge into a continuously used chimney, multiple ultraviolet light barriers across the main air duct, or continuous washing of the discharged air with antiseptic sprays would undoubtedly accomplish this end.

Operation of the isolation unit. Isolation is maintained automatically and continuously by means of the ventilated cubicle system, and it was found unnecessary to observe any precautions in entering the isolation room so long as all cubicles were closed. However, a special technique was used whenever a cubicle was opened and an infected animal was handled; otherwise, infectious material might be transported manually from one cubicle into another. A 2-per-cent aqueous solution of *liquor cresolis compositus* (U. S. P. X.) completely inactivates influenza virus in a few seconds. Consequently, everything that might come in contact with the infected animal was kept constantly wet with this solution. Antiseptic floor traps before each door, a large antiseptic tank, and a pressure spray apparatus[2] (shown in figure 4) were all filled with this solution.

When animals were to be inoculated, examined, or fed, or to have their temperature taken, the operator put on knee-length rubber boots, a full-length rubber coat, heavy rubber gloves, and a gauze face mask. This was done in the vestibule shown in figure 3. All these rubber coverings were then thoroughly wet with the cresol solution. Only one cubicle was opened at a time, and all necessary manipulations were performed without removing the animal from the inner cage. Before the next cubicle was opened, the gloves were again immersed in cresol solution and the coat was washed down with it. Before and after a series of cu-

[2] Made by the DeVilbiss Company, New York City.

bicles was opened, the floor, wall, and racks of the isolation room were sprayed with the cresol solution under pressure.

The cubicles were disinfected by thorough washing, after which they were subjected, on 2 successive days, to vigorous pressure spraying with the cresol solution. The animal cages were sterilized by immersion in boiling water for 30 minutes.

Inoculation was performed in the autopsy room, shown in figure 3, and the animal was immediately placed within a sterile cage inside a clean cubicle which had been brought in for this purpose from the isolation room. The closed cubicle was then returned to its rack and automatically connected with the ventilation system. Following the observation period the closed cubicle was removed from the isolation room and was opened in the autopsy room. Both before and after these procedures the walls and floor of the autopsy room also were sprayed with the cresol solution under pressure.

Effectiveness of isolation. The isolation quarters have been in continuous operation for 6 months. During this period more than 320 ferrets have been isolated in individual cubicles for intervals varying from 10 to 30 days. In every case each cubicle was opened at least once, and usually twice, a day to permit examination and feeding of the animal. Two virus diseases, influenza and distemper, were studied simultaneously in the isolation room. Both of these diseases are readily transmissible by contact in ferrets (Smith, Andrewes, and Laidlaw, 1933; Dunkin and Laidlaw, 1926), and it is well known that distemper is so extremely infectious for ferrets that it is exceedingly difficult to prevent accidental infections.

Approximately 200 ferrets were inoculated with influenza virus. Ten strains of human virus and one strain of swine virus were studied. More than 70 ferrets were inoculated with distemper virus. Two strains, one which had been passed repeatedly in ferrets and another obtained directly from an infected dog, were used. Somewhat over 50 ferrets were not inoculated either with influenza virus or with distemper virus and served as control animals. No attempt was made to separate the various infected and control animals into different groups of cubicles, and

frequently normal ferrets were placed in cubicles adjacent to those containing ferrets in the acute stages of either influenza or distemper.

In no instance did an animal inoculated with influenza virus develop distemper. Similarly, none of the ferrets inoculated with distemper virus became infected with influenza virus, nor did any of these animals develop neutralizing antibodies against the latter virus. Furthermore, none of the control animals contracted either influenza or distemper infections. Serum obtained from each of these animals after varying periods in the isolation room did not in a single instance contain neutralizing antibodies for influenza virus. Finally, a number of the control animals were subsequently tested for susceptibility to either influenza virus or distemper virus. In every instance the animals were found to be fully susceptible and developed infections in every way similar to those observed in normal animals.

It seems evident, therefore, that the isolation achieved in the unit was sufficiently strict to limit either influenza or distemper infections to individual ferrets since no instance of either clinical or inapparent accidental infection occurred.

DISCUSSION

The desirability of facilities which insure the strict isolation of individual experimental animals is obvious. The more infectious a disease and the more readily it is transmitted by contact, the greater is the possibility of accidental infections. In the study of acute upper respiratory disease of human beings isolation of inoculated animals is of fundamental importance, largely because the anatomical peculiarities of the respiratory tract predispose to contact transmission. Studies directed toward the isolation of etiological agents from these diseases are best carried out under conditions which exclude the possibility of accidental infections.

The isolation quarters described were designed to facilitate the study of a relatively large number of animals individually isolated in a reasonably small space. The airtight cubicle system with forced draft ventilation has been shown to operate satisfactorily and to exclude contact infections. It has been possible

to study, simultaneously, animals infected with two different diseases, both of which were highly infectious, without finding in a single instance any evidence of cross infection.

The limiting factor in this isolation system is the ability to prevent the manual transport of infectious material from one animal to another during the necessary intermittent examinations. In the case of the two virus diseases which have been studied it was found that the antiseptic solution used was thoroughly effective in destroying whatever material contaminated the surfaces which had contact with the animal (i.e., gloves, thermometers, etc.). It is possible, however, that this solution might not be effective in the case of very stable viruses, but it is probable that an effective inactivating agent could be found even in the latter instances. In the absence of such an agent the system should still be effective if a fresh pair of sterile gloves were worn when each individual animal was handled.

SUMMARY

Equipment for the individual isolation of infected animals in a single room has been designed and constructed. Animals ranging in size from the mouse to the dog and monkey have been successfully isolated in the unit. Animals are kept in cages which are enclosed in relatively airtight metal cubicles. The cubicles are ventilated by forced draft, and the air entering and leaving each cubicle is filtered. The cubicles can be disconnected from the ventilation system and removed to another room without being opened. The unit was designed primarily for the study of influenza virus in ferrets and has been found to be thoroughly effective in preventing accidental or contact infections. Isolation was sufficiently complete to permit the study of normal ferrets and ferrets infected with either distemper virus or influenza virus in adjacent cubicles without the occurrence of cross infections.

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THE SOIL AS A SOURCE OF MICROORGANISMS ANTAG-
ONISTIC TO DISEASE-PRODUCING BACTERIA[*,1]

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Bacteria pathogenic for man and animals find their way to the soil, either in the excreta of the hosts or in their remains. If one considers the period for which animals and plants have existed on this planet and the great numbers of disease-producing microbes that must have thus gained entrance into the soil, one can only wonder that the soil harbors so few bacteria capable of causing infectious diseases in man and in animals. One hardly thinks of the soil as a source of epidemics. What has become of all the bacteria causing typhoid, dysentery, cholera, diphtheria, pneumonia, bubonic plague, tuberculosis, leprosy, and numerous others? This question was first raised by medical bacteriologists in the eighties of the last century. The soil was searched for bacterial agents of infectious diseases, until the conclusion was reached that these do not survive long in the soil. It was suggested that the cause of the disappearance of these disease-producing organisms in the soil is to be looked for among the soil-inhabiting microbes, antagonistic to the pathogens and bringing about their rapid destruction in the soil.

Pasteur (Pasteur and Joubert, 1877) deserves the credit for having first shown, in 1877, that the production of anthrax in susceptible animals can be repressed by the presence of other microorganisms (". . . on peut introduire à profusion dans un animal la bactéridie charbonneuse sans que celui-ci contracte le

charbon: il suffit qu'au liquide qui tient en suspension la bac-téridie on ait associé en même temps des bactéries communes"). It was soon established that when anthrax, typhoid, staphylo-coccus, diphtheria, and other bacteria are added to the soil they are rapidly antagonized by the soil microbes. Such common sap-rophytes as *Bacterium fluorescens* and *Bacterium pyocyaneum* were found (Garré, 1887; Bouchard, 1889; Freudenreich, 1888) to produce substances, antagonistic to the colon-typhoid group of bacteria as well as to many others. The degree of inhibition varied with the organisms antagonized. Frost (1904), who was among the first to make a detailed study of the rôle of soil organ-isms in repressing the development of pathogens, found that the saprophytes produce substances which not only inhibit the growth of the pathogens, but bring about their destruction. The nature and activity of these thermostable substances were found to depend not only upon the nature of the antagonist, but also upon the specific strain, the composition of the medium and the conditions of growth, especially aeration.

An extensive literature has accumulated on this subject. The numerous antagonistic organisms so far described can be divided into four distinct groups:

The first group comprises bacteria belonging to *Pseudomonas aeruginosa* (*Bacterium pyocyaneum*), *Pseudomonas fluorescens*, and related forms. Marked differences in ability to antagonize bacteria appear to exist among the various strains. The specific nature of the active substance has received a great deal of con-sideration, various claims having been made that it was an enzyme, a pigment, or a lipoid. Hettche (1934) has shown that it passes through collodion membranes, as well as through Seitz and other filters, and is of a lipoid type.

The second group of antagonists comprises various spore-forming bacteria, belonging to the *Bacillus mycoides* and *Bacillus mesentericus* groups. These organisms were found (Pringsheim, 1920; Much and Sartorius, 1924) to bring about lysis of a number of pathogenic bacteria, including diphtheria, typhoid and cholera. In most cases, the antagonistic organisms seemed to be highly specific, acting only upon certain bacteria and not upon others. Recently, Dubos (1939) isolated from the soil an organism belong-

ing to the spore-forming bacteria, which produces a substance capable of destroying gram-positive bacteria; the active agent was crystallized and shown to be a protein derivative. Hettche and Weber (1939), on the other hand, reported the isolation of an active lipoid from spore-forming bacteria. This substance appears to be far more specific than that obtained by Dubos.

A third group of antagonists comprises certain actinomycetes. Some of these organisms inhibit the multiplication of bacteria, whereas others are capable of lysing dead and living bacteria (Lieske, 1921; Gratia and Dath, 1924–1926; Welsch, 1937–1939; Borudulina, 1935). An active substance, which seemed to be largely antagonistic against gram-positive bacteria, was produced (Gratia and Dath, 1924–1926; Nakhimovskaia, 1937). Although this substance was designated as "actinomycetin" (Welsch, 1937–1939), it is fairly certain that different species produce substances which vary in their action.

A fourth group of microorganisms, the fungi, also comprises a number of forms which produce substances active against bacteria, such as "penicillin" obtained by Fleming (1929), and against fungi, as in the case of *Trichoderma* species (Weindling, 1932–1936).

This brief survey is sufficient to emphasize the fact that various microorganisms are capable of forming substances which either inhibit bacterial growth or are bactericidal, or both. These substances vary greatly in composition, in the nature of the organisms which they antagonize, and in the mechanism of the process of inhibition or bactericidal action. Most of the antagonistic organisms appear to act upon gram-positive bacteria, although many also antagonize gram-negative forms. We report certain preliminary results of a study of the nature and abundance of some soil microorganisms, with special reference to their action against various bacteria, comprising both gram-positive and gram-negative forms.

METHOD OF DEMONSTRATING ANTAGONISTIC MICROORGANISMS IN SOIL

The following method has been developed for determining the presence in the soil of microorganisms capable of antagonizing

various specific bacteria. Agar is washed in distilled water and dissolved so as to give 1.5 per cent concentration. Two grams of K_2HPO_4 are added per liter. Ten-milliliter portions of this agar are distributed in test tubes and sterilized. A washed suspension of the specific bacteria, obtained by cultivation on solid or in liquid nutrient media is prepared and added to the washed agar, which has previously been melted and placed in a water bath at 42°C. One-milliliter portions of the still viable bacterial suspension are added to the agar tubes. The bacteria are thoroughly mixed with the agar.

The soil to be tested for the presence and abundance of the antagonists is suspended in sterile tap water, using a series of dilutions, from 1:10 to 1:10,000. One-milliliter portions of these dilutions are placed in sterile Petri dishes and the bacterial agar prepared by the above procedure is added. The plates are well shaken, to distribute the soil suspension thoroughly, and incubated at 28° or 37°C. The presence of antagonists can be demonstrated by the formation of clear zones surrounding the colonies of the latter, after 1–10 days' incubation of the plates. These colonies can now be transferred to fresh bacterial agar plates and later isolated in pure culture by the use of convenient media (figs. 1 and 2).

In the case of certain soils or of specific bacteria, antagonists may not be present at all or they may be there only in limited numbers, not sufficient to give distinct colonies on the plate. Recourse may then be had to the soil enrichment method. Washed bacterial suspensions, for which antagonists are to be obtained, are added, at frequent intervals, to a fresh garden or field soil, kept in beakers or pots under optimum conditions of moisture (60 to 65 per cent of the water-holding capacity), aeration and temperature (28° or 37°C.). The soil is tested from time to time until antagonists can be demonstrated by the plating method.

Attention may be directed here to the fact that the measurement of the concentration of bacteriophage in bacterial cultures is based upon the use of suspensions of live bacteria in agar. Lieske (1921) and Gratia and Dath (1924–1926) suspended

bacteria in agar in order to obtain antagonistic microorganisms, especially actinomycetes; however, they first killed the bacteria
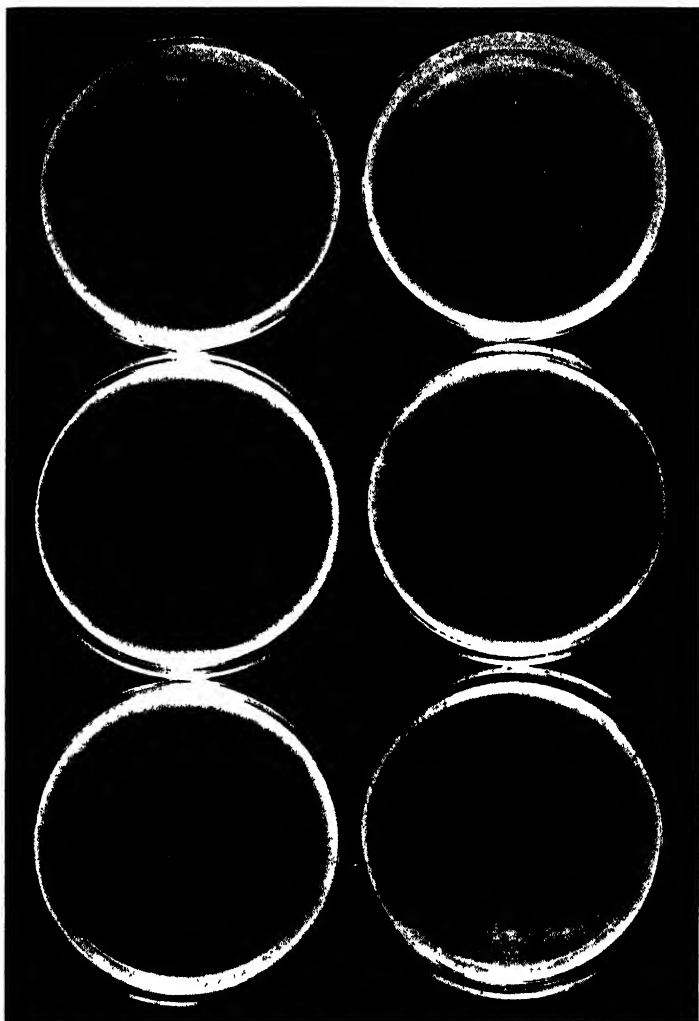
FIG. 1. METHOD OF ISOLATION OF ANTAGONISTIC MICROORGANISMS FROM SOIL
E. coli used as the enriching agent Plates one day old

by the use of heat or chemicals, and exposed the plates to the air in order to catch chance antagonists in the dust. Schiller (1924–

1925) stimulated various organisms to develop antagonistic properties by using specific bacteria as the sole source of nitrogen.

By means of the method outlined here, it was possible to demonstrate that ordinary soils contain a number of microorganisms which are antagonistic against various bacteria, including both gram-negative and gram-positive forms.   The number of antag-
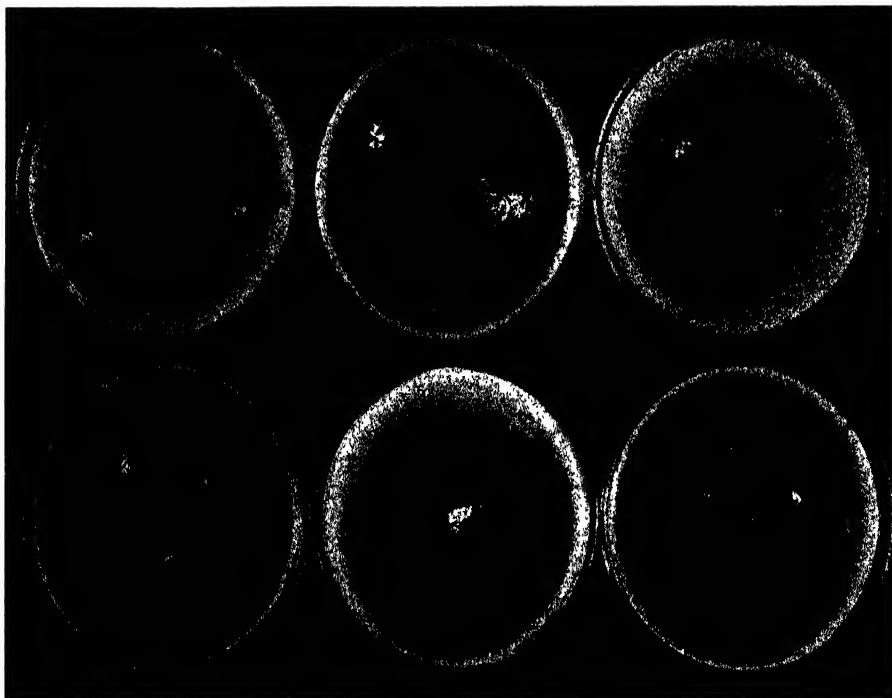
FIG. 2. ISOLATION OF ANTAGONISTS FROM PLATES
Left two plates—first isolation.   Middle two plates—nearly complete clarification.   Two plates at right—reisolation of cultures.

onists was greatly increased in the soil by gradual enrichment with live cells of the specific bacteria which were to be antagonized. A number of antagonistic organisms were isolated.

This report will be limited, however, to a discussion of only two antagonists: (1). a gram-negative organism representing a strain of *Pseudomonas aeruginosa;* (2). a species of *Actinomyces* be-

longing to the chromogenous type, producing black pigments on protein media.

ISOLATION OF ANTAGONISTIC MICROORGANISMS FROM SOIL

In order to demonstrate the effect of enrichment of soil with specific bacteria upon their disappearance and upon the accumulation of organisms antagonistic to them, the results of one experiment are reported (tables 1 and 2). *Escherichia coli* was used for enrichment purposes. The physical and chemical soil conditions had no injurious effect upon this organism, since it

TABLE 1

Survival of Escherichia coli in sterile and in non-sterile soil

Thousands per gram of soil

STERILE SOIL			FRESH SOIL		
Incubation (28°C.)	E. coli	Incubation (28°C.)	No lime	CaCO₃	No lime
days		days	28°C.	28°C.	37°C.
0*	2.6	0	Few§	Few	Few
10	149,000	5†	6,800	3,500	4,700
26	138,000	33†	130	140	10
		127‡	0	1.1	0

* 2,600 cells of *E. coli* added per 1 gram of sterile soil.

† One enrichment before second count; five enrichments before third count.

‡ Eleven enrichments before fourth count, ten days elapsing between last enrichment and plating.

§ Very few colon-like cells in fresh soil (less than 10,000 per gram, practically all of the *Aerobacter aerogenes* type).

not only survived in sterile soil but actually multiplied there at a very rapid rate. The addition of several hundred living cells of *Escherichia coli* to 1 gram of sterile soil (260,000 per 100 grams of soil) resulted in an increase, in 10 days at 28°C., to 149 millions per gram of soil. This tends to prove that the soil, freed from other organisms, is a rather favorable medium for the multiplication of *Escherichia coli*, and that some of the soil organic matter is available to this organism as a source of energy, or may have been made available by the sterilization of the soil. However, the enrichment of fresh soil with large numbers of living *Esch-*

erichia coli cells led to their rapid disappearance; the rate of their destruction increased with every subsequent addition of fresh bacterial cells. The destruction of the *Escherichia coli* was brought about by the development of certain antagonistic microbes which began to multiply rapidly in the enriched soil (table 2). The high number of antagonists even in the control soil may have been partly due to contamination of some of this soil kept in the laboratory, with *Escherichia coli* cells, which resulted in an enrichment of antagonists. When fresh field soils were tested, a much smaller number of antagonists was found in one and only a

TABLE 2

Influence of enrichment of soil with Escherichia coli upon the abundance of antagonists

INCUBATION	CONTROL SOIL	ENRICHED SOIL	ENRICHED SOIL + CaCO₃
Numbers of antagonists* per 1 gram of soil			
days			
65	500,000	4,000,000	6,000,000
117	1,150,000	5,700,000	4,700,000†
Total numbers of microorganisms capable of developing on agar plate			
117	9,100,000	40,000,000	36,300,000

* An antagonistic colony is one surrounded by a halo on the *E. coli* plate.

† This container received fewer enrichments with *E. coli* than the one without CaCO₃.

few in another. The total number of bacteria in the enriched soil, as determined by the plate method, increased very greatly, because the cells of *Escherichia coli* served as good nutrients for many of the soil microorganisms, especially the antagonists.

Several of the organisms isolated were active against the coliform group of bacteria and *Brucella abortus*, in addition to various gram-positive bacteria. Particular emphasis was laid upon a member of the *Pseudomonas aeruginosa* group and a species of *Actinomyces*. These two organisms were grown on a peptone solution (1 per cent Bacto tryptone + 0.5 per cent NaCl), in shallow layers, at 37°C. and 28°C. respectively, for 5 to 10 days.

In the case of the *Actinomyces*, it was sufficient to filter the culture through paper; in the case of the bacterium, a Seitz filter was used to obtain bacteria-free preparations. These were added, in varying concentrations, to sterile nutrient agar media and the inhibiting effect upon various bacteria was tested.

Table 3 shows the inhibiting and bactericidal effect of *Pseudomonas aeruginosa* upon the growth of two test bacteria. *Escherichia coli* was killed rapidly, as demonstrated by streaking the culture on an Endo plate, even when inoculated 1–3 days before

TABLE 3

Bactericidal effect of Pseudomonas aeruginosa upon Escherichia coli and Aerobacter aerogenes

CULTURE	PRELIMINARY INCUBATION*	P. AERUGINOSA†	SURVIVAL OF		
			E. coli	A. aerogenes	P. aeruginosa
	days				
E. coli	0		+		
+	0	+	0		+
+	1	+	0		+
+	2	+	Trace		+
+	3	+	0		+
A. aerogenes	0			+	
+	0	+		Trace	+
+	1	+		Trace	+
+	2	+		Trace	+
+	3	+		Trace	+

* Incubation of cultures, at 37°C., before inoculation with antagonist.
† Total incubation, at 37°C., 6 days; 0 = no growth; + = positive growth.

the antagonist was added to the culture. The destructive effect upon *Aerobacter aerogenes* was not as marked. When the Seitz filtrate of *Pseudomonas aeruginosa* was added to sterile nutrient agar (table 4), it completely inhibited the growth of *Sarcina lutea* in concentration of 1:20 and reduced it in 1:100 concentration; it inhibited the growth of *Bacillus mycoides* and of *Brucella abortus* in 1:10 concentration. However, the filtrate did not inhibit the growth of *Escherichia coli*, even in 1:5 dilution, but merely reduced it somewhat. Repeated experiments demonstrated definitely that, although the presence of living cells of

Pseudomonas aeruginosa was sufficient to inhibit the growth and even kill *Escherichia coli*, the bacteria-free filtrate had no effect upon this organism, although it had a decided effect upon the other three bacteria tested.

An attempt was now made to obtain the active substance in concentrated form. Various reagents were used for extraction

TABLE 4

Inhibiting effect of Seitz filtrate of Pseudomonas aeruginosa upon the growth of four test bacteria*

AMOUNT OF FIL-TRATE ADDED TO 10 ML. AGAR	E. COLI	B. MYCOIDES	S. LUTEA	B. ABORTUS
cc.				
0	3	3	3	3
0.1	3	3	1	3
0.5	3	3	0	3
1.0	3	0	0	0
2.0	2	0	0	

* 0 = no growth, 1 = trace, 2 = fair, 3 = good growth.

TABLE 5

Inhibiting effect of the ether-soluble fraction of Pseudomonas aeruginosa upon the growth of three test bacteria

AMOUNT ADDED TO 10 ML. NUTRIENT AGAR	E. COLI	B. MYCOIDES	S. LUTEA
cc.*			
0	3†	3	3
0.05	3	3	3
0.10	3	3	0
0.50	1	0	0
2.00	0	0	0

* 1 cc. contained 0.4 mgm. of active fraction.
† 0 = no growth, 1 = trace of growth, 2 = fair growth, 3 = good growth.

and concentration, until it was demonstrated that ether extracted most of the active substance. This fraction was found to be highly effective in inhibiting the growth of *Sarcina lutea*, *Bacillus mycoides* and *Escherichia coli*, in increasing concentrations (table 5). It inhibited the growth of *Sarcina lutea* in 1:100 dilution (0.04 mgm. of crude active substance per 10 ml. agar), of *Bacillus*

mycoides in 1:20, and of *Escherichia coli* in 1:5 dilution (0.8 mgm. of substance per 10 ml. agar); it reduced considerably the growth of *Escherichia coli* in a 1:20 dilution (figs. 3 and 4).

The results of a study of the effect of the composition of the medium upon the production of the active substance are given in table 6. The reaction of the medium, as a result of the growth of
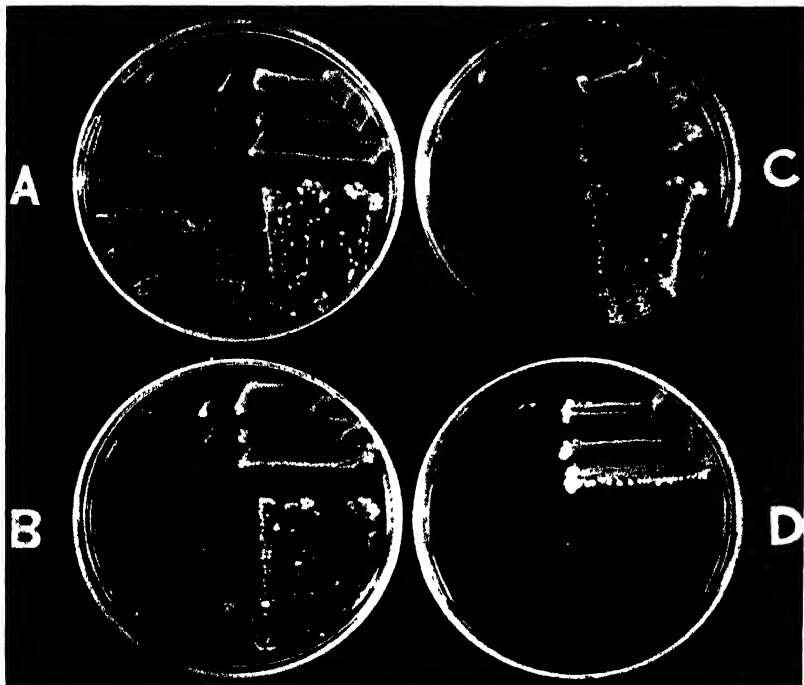
Fig. 3. INFLUENCE OF ETHER-SOLUBLE FRACTION OF P. AERUGINOSA UPON THE GROWTH OF FOUR BACTERIA

A – Control. B, C, D—0.05, 0.2 and 1.0 cc. per plate, respectively. Upper right –*E. coli*, upper left – *B. abortus;* lower right—*B. mycoides;* lower left – *S. lutea.*

Pseudomonas aeruginosa, changed from neutral to pH 9.0; the presence of glucose increased the growth of the organism but did not affect the final pH value. The addition of glucose to the culture reduced its effectiveness; however, it increased the activity of the ether-soluble substance. This is due to the fact that the whole culture, including the thick, slimy growth mass, was ex-
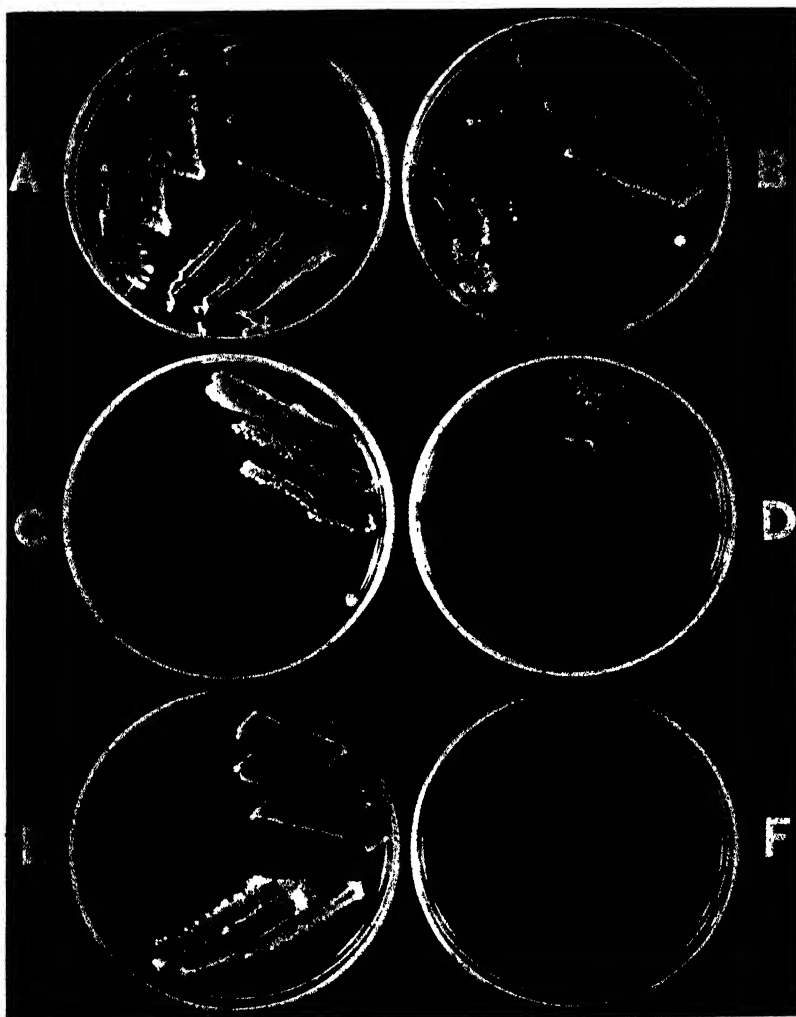
FIG. 4. COURSE OF FORMATION OF ACTIVE ETHER-SOLUBLE SUBSTANCE BY PS.
AERUGINOSA

A—Control; B—1 day incubation, 2 cc.; C, D—2 days incubation, 0.5 and 2.0
cc.; E, F—7 days incubation, 0.1 and 2.0 cc.   All except E: upper right—*E. coli;*
upper left—*B. mycoides;* lower—*S. lutea.*   E: upper right—*E. coli;* upper left
S. lutea; lower—*B. mycoides.*

tracted with ether; a good deal of the active substance was
retained by the cells, growth being heavier on the glucose-con-
taining media.   In the presence of *Escherichia coli*, which made an

effective growth for 24 hours, *Pseudomonas aeruginosa* did not develop so well, especially in the presence of glucose; this is due to the fact that *Escherichia coli* used up a large part of the nutrients and changed the reaction of the medium to acid, in the presence of glucose. As a result of this, in spite of the fact that the *Escherichia coli* was killed, the antagonist produced only a

TABLE 6

*Influence of composition of medium upon the formation of the inhibitory substance by Pseudomonas aeruginosa**

Tryptone broth as basis

ORGANISM	GLUCOSE ADDED	FINAL pH	AMOUNT OF FILTRATE USED	ACTIVITY‡ OF FILTRATE				ACTIVITY‡ OF ETHER SOLUBLE FRACTION		
				S. lutea	*B. mycoides*	*E. coli*	*B. abortus*	*S. lutea*	*B. mycoides*	*E. coli*
			cc.							
P. aeruginosa	0	8.8	0.5	1	3	3	3	0	0	3
P. aeruginosa	0	8.8	1 0	0	0	3	0	0	0	2
P. aeruginosa	+	8 5	0.5	2	3	3	2	0	0	3
P. aeruginosa	+	8 5	1 0	0	1	3	2	0	0	0
E. coli† + P. aeruginosa..	0	8 9	0.5	3	3	3	3	0	2	3
E. coli + P. aeruginosa	0	8.9	1 0	3	3	3	3	0	0	3
E. coli + P. aeruginosa	+	4 5	0 5	3	3	3	3	0	2	3
E. coli + P. aeruginosa	+	4.5	1 0	3	3	3	3	0	1	3

* Active substance 20 times more concentrated in ether-soluble fraction than in original filtrate.

† Inoculated with *E. coli* 24 hours before inoculation with *P. aeruginosa*, incubated at 37°C.; total incubation period 7 days.

‡ 0 = no growth, 1 = trace of growth, 2 = fair growth, 3 = good growth.

limited amount of active substance; the latter could be demonstrated only in the ether-soluble fraction.

The inhibiting action of the *Actinomyces* sp. upon the growth of the three test bacteria was similar in nature but greater in effect (table 7). The paper filtrate of the culture inhibited the growth of *Sarcina lutea* in a dilution of 1:200, of *Bacillus mycoides* in 1:100, but did not inhibit the growth of *Escherichia coli*. Heating the filtrate for 30 minutes at 100°C. did not reduce its

activity.   The ether-soluble fraction, in very great dilution, completely inhibited the growth of all the test organisms.   This active fraction may be designated as *actinomycin,* in contradistinction to the water-soluble actinomycetin of Gratia (1924–1926) and Welsch (1937–1939).

Further studies brought out the fact that many of the actinomycetes of the soil are antagonistic to different bacteria. Some were found to be more effective against the colon group of bacteria, while others were more effective against the *Brucella* group.   It was also demonstrated that the coliform group could

TABLE 7

Inhibiting effect of Actinomyces sp. upon the growth of three test bacteria

AMOUNT ADDED TO 10 ML. NUTRIENT AGAR	PAPER FILTRATE OF ACTINOMYCES SP. CULTURE			HEATED FILTRATE OF* ACTINOMYCES SP. CULTURE			ETHER-SOLUBLE FRACTION[†] OF ACTINOMYCES SP. CULTURE		
	E. coli	*B. mycoides*	*S. lutea*	*E. coli*	*B. mycoides*	*S. lutea*	*E. coli*	*B. mycoides*	*S. lutea*
cc.									
0	3[‡]	3	3	3	3	3	3	3	3
0.01	3	3	3						
0.05	3	3	0						
0.10	3	0	0						
0.50	3	0	0	3	0	0	0	0	0
2.00	3	0	0	3	0	0	0	0	0

* Heated for 30 minutes at 100°C.
† 1 cc. = 0.4 mgm. of active substance.
‡ 0 = no growth, 3 = good growth.

be differentiated on the basis of the action of the antagonist: *Escherichia coli* was most readily acted upon, *Aerobacter aerogenes* least readily and the intermediate strains came between, as shown by the following summary[2]:

ACTINOMYCIN ADDED TO 10 ML. NUTRIENT AGAR	GROWTH OF		
	E. coli	Intermediate	*A. aerogenes*
mgm.			
0.4	0	0	2
0.8	0	0	0

[2] The authors are sincerely indebted to Griffin and Stuart (1940) for supplying these cultures.

The specific action of the antagonists against these organisms may offer an explanation for the common observation that *Escherichia coli* dies out rapidly when added to the soil, whereas *Aerobacter aerogenes* becomes established in the soil.

The inhibiting action of the active ether-soluble substance isolated from the antagonistic Actinomyces grown on agar media was found to be, in the case of the three test organisms, as follows: A dilution of 1:2,500,000 inhibited the growth of *Sarcina lutea* and of *Bacillus mycoides*, and 1:25,000 inhibited the growth of *Escherichia coli* (fig. 5).[3]

Bactericidal action of active substances. Attention has already been called to the fact that the two antagonists studied here have a marked bactericidal effect upon various gram-positive and gram-negative bacteria. This action is not limited to the presence of living and multiplying cells of the antagonists, but is also brought about by the formation of certain bodies which can be isolated from solid and liquid cultures of the antagonists. These active bodies are adsorbed by charcoal and are dissolved by ether. By the use of the latter, followed by treatment with alcohol, then with water, an aqueous solution of the active substances is obtained. This solution had a marked bactericidal effect upon *Escherichia coli* and *Brucella abortus*, as brought out in table 8. The tests were made by adding various dilutions of the active substance to 10 ml. of sterile water containing a suspension of living *Escherichia coli* cells grown on agar media, removed, centrifuged, washed with water and again centrifuged. The apparent increase in the number of cells as a result of addition of very dilute solutions of the active substance may be due to the separation of the flocculated material which otherwise tends to settle to the bottom.

Similar tests were made with *Brucella abortus*. A suspension containing 34,000,000 viable cells was treated with varying

[3] Further studies brought out the fact that the inhibition of the growth of *Staphylococcus albus*, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Streptococcus hemolyticus* and the pneumococcus was of the same range as of *Sarcina lutea*; the inhibition of species of *Neisseria* and of *Mycobacterium tuberculosis* was of the range of *Brucella*, whereas that of the typhoid-paratyphoid group was of the range of *Escherichia coli*.
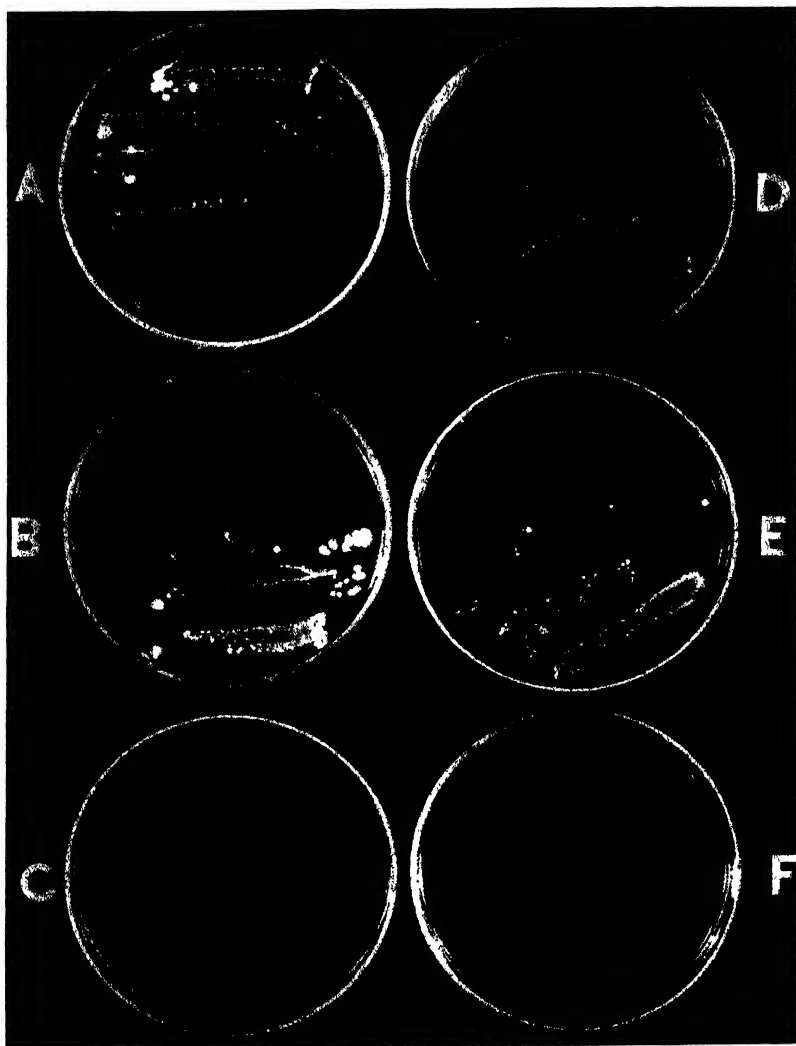
FIG. 5. INHIBITING EFFECT OF ACTINOMYCIN UPON THE GROWTH OF VARIOUS
BACTERIA

A, D—Control; B, E—1 cc.; C, F—2 cc. of actinomycin suspension per plate.
A, B, C: Upper—*B. abortus;* lower—*A. aerogenes.*   D, E, F: Upper right—*E. coli;*
upper left—intermediate; lower—*A. aerogenes.*

amounts of the crude active substance isolated from the antag-
onists.   One ml. of the actinomycin containing about 0.4 mgm.
of the crude active substance killed all cells in 24 hours; it also

killed in that period of time, all the cells in twice the above concentration (68 million viable cells); however, when five times the

TABLE 8

Bactericidal effect of various concentrations of the active substance of two antagonists upon Escherichia coli

Millions of viable bacteria*

NATURE OF ANTAGONIST	AMOUNT OF ACTIVE SUBSTANCE	TIME OF ACTION			
		3 hours	7 hours	24 hours	48 hours
	ml.†				
0	0	216	231	184	238
Actinomyces sp	0.01	224	264	233	154
Actinomyces sp	0 10	250	196	91	22
Actinomyces sp	1.00	126	44	0	0
P. aeruginosa	0.10	265	276	248	275
P. aeruginosa	1.00	177	205	81	93

* As determined by plating on Endo medium.
† 1 ml. = 0.4 mgm. of crude active substance.

TABLE 9

Bactericidal effect of the active substance upon growing cultures of the test organisms

GROWTH OF CULTURE BEFORE ADDITION OF ANTAGONIST	ACTION OF ANTAGONIST											
	24 hours*						48 hours*					
	Actinomyces			*P. aeruginosa*			*Actinomyces*			*P. aeruginosa*		
	E. coli	*B. my-coides*	*S. lutea*	*E. coli*	*B. my-coides*	*S. lutea*	*E coli*	*B. my-coides*	*S lutea*	*E. coli*	*B. my-coides*	*S. lutea*
hours												
Control	3†	3	3	3	3	3	3	3	3	3	3	3
0	0	0‡	0	3	0‡	0	0	0‡	0	3	0‡	0
3	0	0	0	3	0	0	0	0	0	3	0	0
6	0	0	0	3	0	0	0	0	0	3	0	0
22	3	1	0	3	1	3	0	0	0	3	0	1

* Since first inoculation, namely 0 hours.
† 0 = sterile, 1 = trace of growth, 3 = excellent growth.
‡ Some colonies formed on streak due to survival of spores.

concentration was used, not all the *Brucella* cells were killed unless double the concentration of the antagonist was used. The active substance of *Pseudomonas aeruginosa* was less effective.

A study was now made of the effect of the active substance upon actively growing cultures of the three test organisms. One-milliliter portions of the actinomycin (0.4 mgm.) and bacterial substance were added to 10 ml. nutrient broth inoculated with the different organisms. The results (table 9) show that the actinomycin killed *Sarcina lutea* and *Escherichia coli*, as well as the vegetative cells of *Bacillus mycoides* (spores remaining unaffected), when added at the start, and after 3, 6 and 22 hours inoculation with the test organisms. The bactericidal action was demonstrated by streaking the culture on nutrient agar. The substance obtained from *Pseudomonas aeruginosa* was less effec-

TABLE 10

Effect of the active substance of two antagonists upon the bacteria in fresh milk

Bacterial numbers in 1 ml. milk

NATURE OF ANTAGONIST	AMOUNT OF ACTIVE SUBSTANCE ADDED TO 10 ML. FRESH MILK	TIME OF INCUBATION	
		6 hours	16 hours
	ml.		
0	0	240,000	485,600,000
Actinomyces sp.	0.1	60,000	
Actinomyces sp. . . .	1.0	15,000	
Actinomyces sp.	1 0		18,600,000
P. aeruginosa	0.1	160,000	.
P. aeruginosa . .	1.0	5,000	

tive, its action being limited, killing only the *Sarcina lutea* and the vegetative cells of *Bacillus mycoides*, but not affecting *Escherichia coli*.

In order to illustrate the selective bactericidal effect of the two active substances upon a mixed bacterial population, varying amounts of these substances were added to 10-ml. portions of fresh milk. These were incubated, for 6 and 16 hours, at 28°C. and counts made, by plating on nutrient agar. The results (table 10) show that both active substances had a marked effect, even in very low concentrations, in killing and in inhibiting the multiplication of certain types of bacteria in the milk. When

0.1 and 1.0-ml. portions of actinomycin were added to 10-ml. portions of nutrient agar and fresh soil plated out, using these media, the reduction in the number of bacterial and actinomyces colonies was 97–99 per cent for the lower concentration and 100 per cent for the higher.   The fungi of the soil did not seem to be affected.

SUMMARY

The soil contains a number of different types of microorganisms antagonistic to various bacteria belonging to the gram-positive and gram-negative groups.   By enriching the soil with the specific bacteria, the corresponding antagonists increase and can be readily isolated.   This has been done most readily by the use of an agar medium containing viable cells of the specific organism as the sole available nutrient.

Out of a number of antagonistic organisms isolated from the soil, two were studied in greater detail, one a bacterium belonging to the *Pseudomonas aeruginosa* group, and the other an *Actinomyces*.   These organisms were found to inhibit the growth of several gram-negative bacteria, as well and, even to a greater extent, of numerous gram-positive bacteria.

The active substance produced by the two antagonists was found to be largely thermostable; it passed through a Seitz filter, it was removed by charcoal and was, partly at least, ether-soluble.   Highly active preparations were obtained which inhibited, in very dilute solutions, the growth of *Escherichia coli*, *Brucella abortus*, and of many other bacteria.

The active substance had also a strong bactericidal effect upon *Escherichia coli* and *Brucella abortus*; 1 ml. of the preparations containing 0.4 mgm. of the crude active substance killed aqueous suspensions of *Escherichia coli* (215,000,000 viable cells) and of *Brucella abortus* (68,000,000 viable cells).

The active substance of the two antagonists was found to reduce, in very low concentrations, the bacterial population of natural substrates, such as milk; when added to agar it prevented the development of the great majority of soil bacteria and actinomycetes, but not of fungi.

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THE PRODUCTION OF STAPHYLOCOCCAL ALPHA-HEMOLYSIN: THE RÔLE OF AGAR[1]

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For the production of high titer staphylococcus toxin,[2] the addition of agar (Burnet 1930) to the medium, and incubation under an atmosphere containing added carbon dioxide (Parker, Hopkins and Gunther, 1925–1926) have been found to be essential in most instances. Shallow layers of semi-solid nutrient agar (0.3 to 0.5 per cent) are inoculated with the staphylococcus and incubated under an atmosphere containing measured amounts of carbon dioxide in oxygen or air. Incubation is continued as a rule for 48 to 72 hours and the toxin is obtained after removal of the agar and organisms by filtration. Toxin produced by this procedure has been of much greater potency than that formed in fluid media.

Walbum (1922), studying the formation of a "hot-cold" hemolysin for goat cells in a fluid medium, noted that for optimum "hemolysin" production the best results were obtained when the medium contained magnesium, from 0.1 to 0.3 per cent peptone and a diminished amount of meat extractives and sodium chloride. The speed of production and deterioration of toxin was greater with the smaller amounts of peptone than with the larger.

McLean (1937) found that by immersing a cellophane bag containing saline, in broth, for 24 hours and then inoculating the contents of the bag with a toxin-producing strain of staphylococcus (Wood 46), he could obtain a toxin of a titer (Lh 0.1) which compared favorably with that produced by the semi-solid agar technique (Lh 0.08). He attributed the increase in toxin

formation through the use of agar and the cellophane bag to the adsorption of substances inhibiting toxin production. The cellophane bag procedure, however, could not be applied to large volumes of medium for the elaboration of potent toxin.

McIlwain (1938) attempted to determine the constituent of agar that was responsible for the increase in toxin production when added to the chemically defined medium of Gladstone (1938). He found that toxin formation was affected unfavorably by the calcium of the agar, and that the remainder of the agar molecule served in a toxigenic manner. The stimulating property was associated with the gelling power of the agar. McIlwain also suggested that this favorable action of agar was due to the adsorption of inhibiting substances.

Attempts were made by the present author to make the gases concerned more available to broth cultures of bacteria without adding such extraneous substances as agar or cellophane to the medium. To accomplish this, two procedures were employed: (1) Bubbling the gases through the culture (Casman 1938) and (2) continuous rocking. With both procedures a marked enhancement of toxin production was obtained.

BUBBLING GASES THROUGH THE CULTURE—METHODS AND MATERIALS

The medium used in the experiments described below was usually veal infusion (500 grams per liter of distilled water) containing 2 per cent proteose peptone (Difco) and 0.7 per cent sodium acetate. Occasionally 0.5 per cent sodium chloride was included. The pH, except where otherwise noted, was 6.8.

For the bubbling of gas through the culture a modification of the apparatus of Plastridge and Rettger (1929) was used. The gas was forced through a trap containing sulphuric acid, then through the culture tube at a rate of approximately 100 ml. per hour. The culture tube measured 1.0 by 8½ inches, and was aerated by means of a glass tube having a bulb at one end. The bulb was perforated with seven or eight small holes. In later experiments a sintered glass gas-diffusing tube was employed. Using this procedure, 50 ml. of medium were inoculated; during

the aeration process samples were removed at various intervals for toxin titration, pH determination and estimation of amount of growth by matching with turbidity standards (McFarland, 1907).[3]

The toxin-producing strain used in the "bubbling" experiments was #2081, obtained from the Sharp & Dohme Laboratories. It was usually added as one drop of a saline suspension (one billion organisms per ml.) of a 24-hour beef-infusion agar slant culture.

The toxin was titrated with staphylococcus antitoxin prepared in horses by the Sharp & Dohme Laboratories at Glenolden, Pa., and checked with standard antitoxin obtained from the National Institute of Health. The Lh values were determined by mixing varying amounts of toxin with a fixed amount of antitoxin (usually one-half international unit). After preliminary incubation at 37.5°C. for $\frac{1}{2}$ hour, indicator cells in the form of an equal volume of 2 per cent washed rabbit erythrocytes were added. The dilution showing 50 per cent hemolysis after another hour's incubation at 37.5°C. was taken as the end point. Lf determinations were sometimes made.

RESULTS

In order to compare different combinations of gases, the following gas mixtures were used in an aeration (bubbling) experiment: A, oxygen; B, 95 per cent O_2 plus 5 per cent CO_2; C, 80 per cent O_2 plus 20 per cent CO_2; D, air. A veal-infusion medium of pH 7.2 containing 1 per cent proteose peptone and prepared according to Wright's method (1933) was employed. Samples were removed and studied after 24, 72 and 120 hours of aeration.

From figure 1 it is evident that, with an initial mixture of 80 per cent O_2 and 20 per cent CO_2, the Lh value at 120 hours was 0.1 ml. Aeration with ordinary air resulted in the production of no appreciable amount of toxin, while the mixture of 80 per cent O_2 and 20 per cent CO_2 was markedly superior to either oxygen alone or a mixture of 95 per cent O_2 and 5 per cent CO_2.

[3] When plate counts were made the values were much less than those obtained by the turbidity-matching procedure. The latter technique was used, however, because of its convenience and probable greater accuracy in determining relative amounts of growth.

Since Walbum (1922) had been able to accelerate toxin pro-
duction by diminishing the sodium chloride, meat extractives and
peptone content of his medium, a study was made of the effect of
dilution of the medium on toxin production while aerating with
80 per cent O_2 and 20 per cent CO_2 by the bubbling procedure.
The basic medium was veal infusion containing 2 per cent proteose
peptone, 0.7 per cent sodium acetate and 0.5 per cent sodium
chloride.   The undiluted medium and portions diluted with
water to give 75, 62.5 and 50 per cent medium concentration were

A Oxygen
B 5% carbon dioxide in oxygen
C 20% "   "   "   "
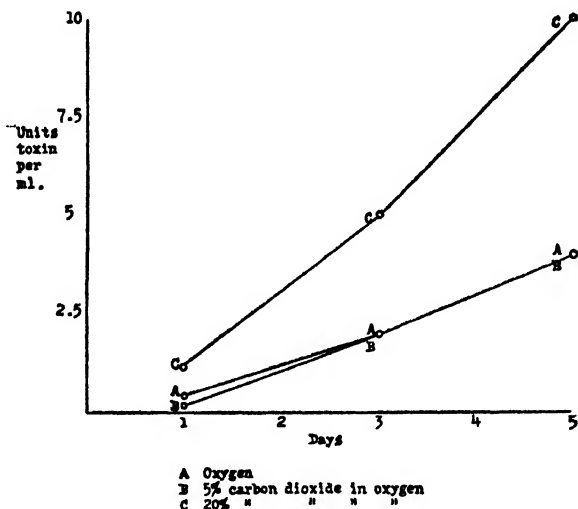
FIG. 1

aerated for 113 hours.   Samples were removed at the end of 24,
48, 72 and 96 hours and titrated for their toxin content.   In
titrating these samples the supernatants obtained after centrifuga-
tion were used.

The pH, Lh, and in some instances the Lf, values are presented
in table 1.   It is evident that by dilution of the medium the
aeration and incubation time may be shortened to 72 and 96
hours and that potent toxin may be obtained (Lh 0.04).   It is
also evident that toxin production is associated with a rise in pH.

When the "bubbling" aeration procedure was applied to a

large volume of medium in a Florence flask, no appreciable amounts of toxin were obtained. It was observed that the gas evolved in the aeration tube would rise almost in a straight line to the surface and escape without aerating the medium. By constant stirring of such a large volume of medium and by aeration with very small bubbles of gas it was possible to obtain an appreciable increase in the amount of toxin. By diluting the basic medium described above with an equal quantity of distilled water, and with the use of a stirring device, it has been possible to obtain an Lh value of 0.1 ml. in 48 hours in a large volume of medium (3500 ml.). A Berkefeld V filter was used in order to obtain bubbles of suitable size.

TABLE 1

Effect of dilution of the medium on toxin production and pH in the "bubbling aeration" procedure

PER CENT MEDIUM	24 HOURS		48 HOURS			72 HOURS			96 HOURS			113 HOURS		
	Lh	pH	Lh	Lf	pH	Lh	Lf	pH	Lh	Lf	pH	Lh	Lf	pH
100	0.5	6.4	.16		6.6	0.1		6.8	.08	.125	7.3	.06	0.1	7.3
75	.24	6.4	0.1	.125	6.7	.06	.08	7.2	<.06	.07	7.5	.06	.075	7.7
62.5	.22	6.4	.09	.09	6.8	.06	.06	7.3	.04	.05	7.6	.06	.045	7.7
50	0.2	6.5	.08	.075	7.0	.06	.065	7.3	<.06	.055	7.7	.06	.055	7.8

In numerous "bubbling" aeration experiments with the diluted veal infusion medium and with media consisting of yeast extract and acid digests of casein or gelatin there was a moderate toxin yield in from 24 to 48 hours. On continued aeration, however, there was a loss of potency. An example of this is evident in table 1 in which the results obtained with diluted veal-infusion medium are presented. Here the Lh values for the diluted media are greater at the end of 113 hours than at 96 hours.

Although toxin production was markedly improved by the "bubbling" aeration procedure, it did not equal that obtained by the agar method (Lh 0.025 to 0.03).

AERATION BY CONTINUOUS ROCKING

In experiments in which aeration by rocking was practiced, veal-infusion medium was employed, as before. Shallow layers

of the medium (5 ml.) in 50 ml. Erlenmeyer flasks were inoculated with one drop of a saline suspension containing approximately one billion organisms per ml. of a washed culture of a suitable strain of staphylococcus. The flasks were closed with sterile hollow rubber stoppers through which sterile hypodermic needles were passed. The hubs of the needles were plugged with cotton. The flasks were placed in a container which was evacuated and then filled with the desired gaseous mixture. The needles were withdrawn from the stoppers, and the flasks attached to a wooden rocker (figure 2) by means of adhesive tape and thumb tacks or by tying them into tin bases which were attached to the rocker by means of screws. The rocking mechanism carried the flasks through an arc of 45 degrees every three seconds.

Four good toxin-producing strains were used: ⚹1, isolated at autopsy from the duodenum of a case of acute hepatocellular disease; ⚹2081, from Sharp and Dohme Laboratories; ⚹24MA, from Connaught Laboratories, Toronto, Canada; Wood 46, from Connaught Laboratories, Toronto, Canada.

Comparison with the agar method was made by adding 0.5 per cent Difco agar to the medium and incubating without rocking. The amount of medium, the size of the flask and the method of filling the flask with the gas mixture were the same with the fluid and the semi-solid agar cultures. In the inoculation of the agar medium 0.05 to 0.1 ml. of bacterial suspension was used. The inoculum was distributed over the surface of the semi-solid medium by careful tipping of the flask.

RESULTS

In order to compare the results of the rocking aeration procedure with those of stationary incubation and to determine to what extent the addition of agar to the medium influenced the production of toxin, an experiment was performed in which media containing 0.03 and 0.5 per cent Difco agar and 0.03 and 0.5 per cent potassium agar (prepared from Difco agar according to the procedure described by McIlwain in 1938) were compared to the same medium minus the agar. The addition of 0.03 per cent agar resulted in no apparent decrease in the fluidity of the

FIG. 2

medium, while the addition of 0.5 per cent agar gave the medium a semi-solid consistency. One set of fluid cultures was rocked continuously, while another was incubated without rocking. The

latter set was gently shaken by hand one or two times daily. The semi-solid cultures were incubated without any rocking or shaking. A gas mixture of 20 per cent carbon dioxide in oxygen was employed.

TABLE 2

Comparison of results obtained with stationary semi-solid agar medium and rocked and stationary liquid (agar-free and low agar) media

STRAIN	AMOUNT OF AGAR	ROCKED		STATIONARY	
		Lh	Growth	Lh	Growth
#1	0.0	0.04	12	0.12	3 5
#1	0.03% Ca	0.036	13 5	0.14	5
#1	0 03% K	0 046	12	0.14	5
#1	0.5% Ca			0 024	
#1	0 5% K			0 02	
#2081	0 0	0.022	9	0 12	3 5
#2081	0 03% Ca	0.022	10 5	0 16	5 5
#2081	0 03% K	0 022	7 5	0 12	3 5
#2081	0 5% Ca			0 026	
#2081	0 5% K			0.04	
#24MA	0 0	0 014	12	0 07	5
#24MA	0.03% Ca	0 018	12	0 08	6
#24MA	0.03% K	0 01	9	0 4	6 5
#24MA	0 5% Ca			0 008	
#24MA	0 5% K			0 008	
Wood 46	0 0	0 016	12	0.08	2 5
Wood 46	0 03% Ca	0.022	18	0.07	4
Wood 46	0.03% K	0 02	18	0 09	4
Wood 46	0 5% Ca			0.024	
Wood 46	0.5% K			0 016	

Note: Ca, ordinary agar containing calcium; K, potassium agar. Growth expressed in billions of organisms per ml.

Following 66 hours of incubation the cultures were harvested. After estimation of the amount of growth and then centrifugation, the supernatants were titrated for their toxin. The results of this experiment are presented in table 2. It is evident that rocking results in an increase in toxin production and growth. The addition of 0.03 per cent agar to the medium did not result

in an increase in toxin production. With two strains (#1 and #24MA) the addition of 0.5 per cent agar resulted in the production of a more potent toxin than was obtained by the "rocking aeration" technique. With strain #2081, however, the rocking procedure proved to be slightly superior to the semi-solid agar technique. The Wood 46 strain cultured on ordinary agar produced less toxin than when grown on the potassium agar. On the latter medium the toxin yield was equal to that obtained by the "rocking aeration" procedure.

TABLE 3

The effect of different gaseous atmospheres on toxin production in fluid and in semi-solid mediums

PER CENT CO₂	STAPH 24MA			STAPH WOOD 46		
	0.7 per cent K agar Lh	Fluid medium		0.7 per cent K agar Lh	Fluid medium	
		Lh	Growth		Lh	Growth
10	0.01	0.014	12	0.02	0.016	15
20	0.01	0.011	9	0.018	0.016	9
36	0.012	0.01	3	0.012	0.016	15
44	0.01	0.011	2.5	0.012	0.012	12
54	0.01	0.012	2.7	0.012	0.012	9
63	0.016	0.016	3	0.014	0.016	4.5
80	0.026	0.05	4	0.09	0.038	1
80R	0.008	0.03	6	0.02	0.034	4.5

Note: Growth is expressed as billions of organisms per ml.
R = gaseous atmosphere renewed at 24 and 48 hours.

Using the "rocking aeration" procedure for fluid cultures and stationary incubation for semi-solid potassium agar, toxin production was studied after incubating the cultures for sixty hours under the following amounts of carbon dioxide in oxygen: 10, 20, 36, 44, 54, 63 and 80 per cent. For incubation in 80 per cent carbon dioxide in oxygen, two sets of cultures were prepared; one set was incubated in the usual continuous manner, while with the other the gaseous atmosphere of 80 per cent carbon dioxide in oxygen was renewed after 24 and 48 hours of incubation.

In table 3 are presented the results obtained with strains 24MA and Wood 46. It was observed that the amount of growth varied

directly with the concentration of oxygen in the atmosphere. While the determination of the amount of growth in the fluid medium was complicated by a tendency of the organisms (especially 24MA) to undergo autolysis, the observed relationship between the amount of growth and the concentration of oxygen was definitely evident in the agar cultures in which less autolysis appeared to take place. Renewal of the gaseous atmosphere of 80 per cent carbon dioxide and 20 per cent oxygen resulted in a definite increase in the amount of growth with both strains and in both types of media.

Examination of the Lh values indicates that with increased carbon dioxide tension there was an increase in toxin production, provided that there was at the time sufficient oxygen for the support of growth. With diminished growth as a result of smaller concentrations of oxygen, there was a diminution in the amount of toxin produced. When the oxygen was renewed in the presence of as much as 80 per cent carbon dioxide, excellent toxin production was obtained. Under the conditions prevailing in this experiment, an atmosphere of 45 per cent carbon dioxide and 55 per cent oxygen appeared to be best for the production of toxin by these strains.

In order to compare the rocking procedure with the semi-solid agar technique under a more optimal gaseous atmosphere, undiluted fluid medium was inoculated and rocked, while the same medium containing 0.5 per cent Difco (calcium) agar or 0.6 per cent potassium agar was subjected to stationary incubation. The gaseous atmosphere was 40 per cent CO_2 in oxygen. The initial pH of the medium was 6.9. Strains #1, #2081, #24MA and Wood 46 were used and the incubation periods were 26, 48 and 68 hours.

The results presented in table 4 again show that with strains 24MA and Wood 46 the rocked broth culture procedure is as good as the semi-solid agar technique for toxin production. With both strains 100 Lh units of toxin per ml. were obtained. With strain #2081 the rocked broth procedure was only slightly inferior to the semi-solid agar technique. Strain #1, however, produced more toxin when the semi-solid agar procedure was used than it

did with the rocked broth technique.   No marked difference between the amounts of toxin production in the calcium and potassium agars was evident.

In order to determine the effect of dilution of the medium on toxin production in the closed system of the "rocking aeration" procedure, the basal medium was diluted with water to give the following concentrations of medium: 100, 75 and 62.5 per cent. To portions of these diluted media 0.5 per cent NaCl was added.

TABLE 4

Comparison of results of "rocked broth" and "semi-solid agar" procedures, using a gaseous atmosphere of 40 per cent CO_2 in O_2

STRAIN	MEDIUM	Lh		
		26 hours	48 hours	68 hours
#1	Broth	0.034	0.034	0.028
#1	Ca agar	0.018	0.016	0.018
#1	K agar	0.018	0.02	0.018
#2081	Broth	0.026	0.022	0.04
#2081	Ca agar	0.022	0.02	0.023
#2081	K agar	0.02	0.02	0.018
#24MA	Broth	0.014	0.01	0.009
#24MA	Ca agar	0.011	0.01	0.01
#24MA	K agar	0.011	0.011	0.048
Wood 46	Broth	0.011	0.01	0.011
Wood 46	Ca agar	0.014	0.012	0.014
Wood 46	K agar	0.011	0.014	0.019

Strains #1, #2081 and #24MA were employed.   For comparison with the semi-solid agar culture procedure, the undiluted basal medium (without added salt) to which 0.6 per cent potassium agar was added was also used.   The gaseous atmosphere was 40 per cent carbon dioxide in oxygen and the incubation periods were 24 and 45 hours.

From table 5 it is evident that after 24 and 45 hours of incubation no improvement in the amount of toxin production was obtained as a result of dilution of the medium.   With strain #1

the semi-solid agar culture technique gave a higher toxin yield than did the rocked fluid culture. With strains #2081 and #24MA the toxin yields with the two methods were about the same.

TABLE 5

Effect of dilution of fluid medium on toxin production, using the rocking procedure

STRAIN	TYPE OF MEDIUM	PER CENT BASAL MEDIUM	PER CENT NaCl	GROWTH		Lh	
				24 hours	45 hours	24 hours	45 hours
#1	Broth	100	0.0	15	15	0.028	0.028
#1	Broth	75	0.0	12	9.5	0.036	0.035
#1	Broth	62.5	0.0	9	9.5	0.04	0.05
#1	Broth	100	0.5	18	18	0.04	0.032
#1	Broth	75	0.5	14	9.5	0.038	0.033
#1	Broth	62.5	0.5	10.5	4.5	0.038	0.035
#1	Agar	100	0.0			0.02	0.014
#2081	Broth	100	0.0	10.5	12	0.024	0.022
#2081	Broth	75	0.0	9.5	9	0.03	0.028
#2081	Broth	62.5	0.0	7.5	6	0.04	0.036
#2081	Broth	100	0.5	14.5	18	0.024	0.042
#2081	Broth	75	0.5	12	9	0.028	0.026
#2081	Broth	62.5	0.5	6	7.5	0.032	0.03
#2081	Agar	100	0.0			0.024	0.034
#24MA	Broth	100	0.0	15	12	0.014	0.011
#24MA	Broth	75	0.0	15	9	0.018	0.018
#24MA	Broth	62.5	0.0	12	9	0.02	0.024
#24MA	Broth	100	0.5	12	12	0.013	0.012
#24MA	Broth	75	0.5	15	9	0.014	0.014
#24MA	Broth	62.5	0.5	13	7.5	0.02	0.021
#24MA	Agar	100	0.0			0.011	0.01

Note: Growth is expressed in billions of organisms per ml.

Since renewal of the gaseous atmosphere resulted in an increased toxin yield (see table 3), an attempt was made to determine to what extent the volume of the gaseous atmosphere influenced toxin production. To this end, 2½, 6- and 12-oz. cylindrical soft glass jars having approximately the same diameters, but varying in height, were used instead of Erlenmeyer

flasks. The 2½ oz. jars measured 2 inches in diameter while the 6 and 12 oz. jars had a diameter of 2¼ inches and differed in height only. To the 2½ oz. jar, 8 ml. and to the 6 and 12 oz. jars,
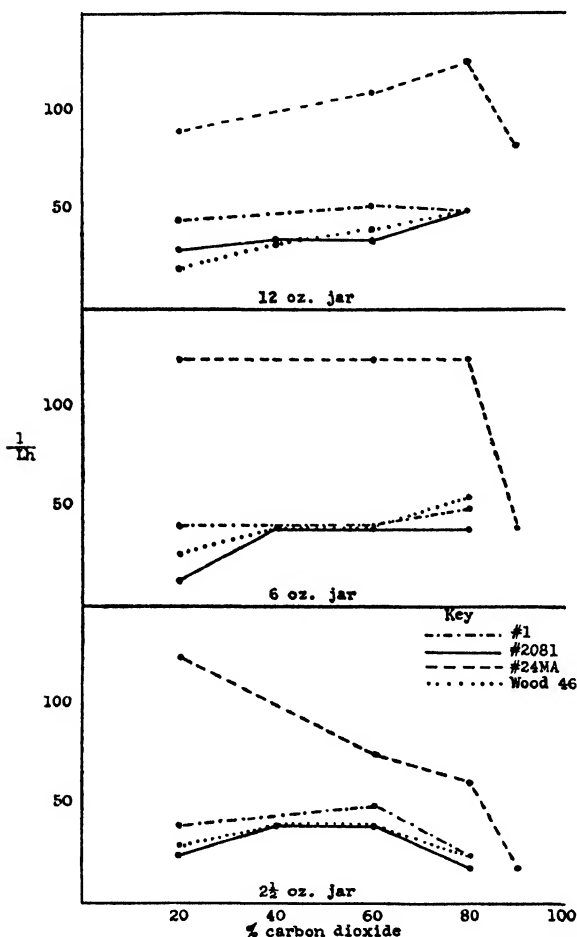
FIG. 3

10 ml. of semi-solid 0.5 per cent Difco agar medium were added (in order to obtain approximately the same surface-area to volume relationship). The medium was inoculated in the usual manner and the jars filled with the different mixtures of carbon dioxide

and oxygen. Lh determinations were made after stationary incubation at 37.5°C. for the following periods of time:

Strain	Hours Incubated
#1	60
#2081	80
#24MA	66
Wood 46	80

The results of these studies are presented in figure 3. It is apparent that the optimum mixture of carbon dioxide and oxygen for toxin production depends on the volume of gas mixture available to the culture. For small volumes, the oxygen to carbon dioxide ratio employed should be greater than for larger volumes of gas. This dependence of toxin production on the volume of available gas mixture is especially noticeable in the results obtained with strain #24MA. In a $2\frac{1}{2}$ oz. jar containing 20 per cent CO_2 and 80 per cent O_2, this strain produced 125 units of toxin per ml. In a 12 oz. jar containing the same gas mixture, 90 units of toxin were produced per ml. On the other hand, when a mixture of 80 per cent CO_2 and 20 per cent O_2 was employed the results were reversed. In a $2\frac{1}{2}$ oz. jar, 60 units of toxin per ml. were produced while in the 12 oz. jar, 125 units per ml. were obtained.

DISCUSSION

The elimination of agar from media used in the preparation of staphylococcus alpha-hemolysin has obvious practical advantages and should facilitate study of the mechanism of toxin production by this organism.

The studies described here indicate that the function of agar is chiefly one of making more available to the staphylococcus the gaseous atmosphere under which it is cultured rather than the adsorption of substances deleterious to the production of alpha-hemolysin. Perhaps in some of the less complex media like that of Gladstone, in which the presence of substances inhibitory to toxin formation may play a greater rôle, agar may act by adsorbing these substances. Our own experiences with such a

medium[4] have been too irregular to permit conclusions in this respect.

McClean's results with the cellophane bag technique may be explained as due to an increased accessibility of the gaseous atmosphere to the bacteria as a result of limitation of growth to the surface or subsurface portions of the broth. Although we have been able to obtain potent toxin with strain #2081 by employing the "cellophane bag procedure," such results have been far from consistent. McClean's use of the Wood 46 strain may account for the consistency of his results. This strain appears to give a better yield of toxin in a shallow layer of fluid medium than do most toxin-producing strains of staphylococcus. The Lh values reported by McClean with both cellophane bag and agar procedures (0.1 and 0.08 ml. respectively) are approximately the same as the value obtained by us in the stationary shallow fluid culture of this strain (see table 2).

Gladstone (1938) suggested that his failure to obtain toxin by the "bubbling aeration" procedure with a chemically defined medium was due to the destruction of the toxin in such a medium by denaturation. He suggested that the discrepancy between his results and ours (Casman 1938) might be due to the protective action exerted by colloids in the veal-infusion proteose-peptone medium used by us. This suggestion is supported by our results with diluted veal-infusion proteose-peptone media and with media prepared with an alcoholic extract of yeast and an acid digest of gelatin (or casein). The superiority of the "rocking aeration" procedure over the "bubbling aeration" procedure may be due to the elimination of this destructive action in the former method.

The studies on the influence of different volumes of combinations of oxygen and carbon dioxide indicate the importance of the gaseous atmosphere and especially of carbon dioxide in the production of staphylococcal toxin. It is interesting to note, in this respect, that while most investigators recommend the use of

[4] A modification of Gladstone's medium using an acid digest of gelatin enriched with cystine, tryptophane, glucose, nicotinic acid, thiamine chloride, Fe, Cu, Mg salts and phosphate.

a mixture of from 20 to 40 per cent carbon dioxide in oxygen or air, Leonard and Holm (1935) employing a relatively large tank for their cultures, found a mixture of 80 per cent carbon dioxide and 20 per cent oxygen most favorable for toxin production by staphylococcus. It is to be borne in mind that the gaseous atmospheres indicated as optimal for toxin production are the initial atmospheres of the closed systems employed. Since oxygen is utilized and carbon dioxide produced as a result of the growth and metabolism of the staphylococcus a concentration of carbon dioxide higher than the initial one is obtained on incubation.

SUMMARY

1. Aeration of fluid cultures of staphylococcus by bubbling gas mixtures through them resulted in an enhancement of toxin production. The toxin so produced did not equal in potency that obtained by the semi-solid agar procedure.

2. The early work of Walbum, in which dilution of the medium was found to accelerate toxin production, was confirmed when the "bubbling aeration" procedure was employed. Such acceleration was not evident in the "rocking aeration" experiments.

3. Aeration of shallow fluid cultures of four strains of staphylococcus by continuous gentle rocking resulted in an even more marked enhancement of toxin production than when the cultures were aerated by bubbling. With three of these strains the toxin produced by the former method equalled in potency the toxin obtained by the semi-solid agar technique.

4. The optimal initial mixture of oxygen and carbon dioxide for the production of alpha-hemolysin in a closed system varies with the total gas volume under which the organism is grown.

CONCLUSIONS

Some of the active alpha-hemolysin-producing strains of staphylococcus elaborate as much alpha-hemolysin by suitable aeration of fluid (agar free) cultures as they do by the use of the semi-solid agar technique.

nical assistance; to Doctor John Eiman, Director of the laboratories, for his support and cooperation; and to the Sharp and Dohme Laboratories of Glenolden, Pa., for the furnishing of liberal supplies of staphylococcal antitoxin.

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STUDIES ON THE CYTOPHAGAS

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· INTRODUCTION

The morphology and taxonomy of the cytophagas have been the subject of much controversy during the last decade. The difficulties of these problems may be judged from the fact that these organisms have been placed in no less than three orders by different workers; the *Spirochaetales* (Hutchinson and Clayton), the *Actinomycetales* (Bokor) and the *Myxobacteriales* (Krzemieniewska).

Van Iterson (1904), the first to demonstrate the aerobic decomposition of cellulose by bacteria, undoubtedly obtained representatives of the cytophagas in his enrichment cultures. In addition to *Bacillus ferrugineus*, which he considered to be the active organism in cellulose decomposition, a large coccus often occurred. This is figured in his paper as covering the cellulose fibers in a manner highly characteristic of the microcysts of *Cytophaga myxococcoides*. Van Iterson's attempts to isolate the different organisms found in enrichment cultures were unsuccessful, and as a result he failed to appreciate the true importance of the "coccus," regarding it as favoring, but not playing an active role in, cellulose decomposition.

In 1919, Hutchinson and Clayton isolated from soil an aerobic cellulose decomposer, characterized by its strict adaptation to cellulose (no other energy source could be used) and by its peculiar morphology. Two morphological types always occurred in their cultures; a long, thin, flexible, pointed rod and a large coccus. In young cultures the rod form predominated, while in older cultures it was replaced by the coccus. All attempts at sepa-

rating these two forms having failed, Hutchinson and Clayton concluded that they were stages in the developmental cycle of a single species, a contention which was supported by the occurrence of cells which could be regarded as transitional types. The organism was placed in the genus *Spirochaeta* with the comment that its peculiar developmental cycle made it impossible to regard it as a representative of the true bacteria, and that some of its characteristics seemed to favor a closer relationship with the spirochaetes. The authors emphasized that:

> ... the organism under consideration exhibits a number of features which have not hitherto been observed in the spirochaetes, features which however appear to indicate a more complex development than that of the true bacteria.

To the coccus form they gave the designation of "sporoid."

Winogradsky (1929), in an extensive investigation of aerobic cellulose decomposition in the soil, isolated and studied a number of organisms which resembled morphologically the vegetative cells of *Spirochaeta cytophaga*. In certain of his crude cultures Winogradsky observed coccus forms but in others, which were otherwise apparently identical, they were not present. Consequently, Winogradsky regarded the cocci as contaminants and always chose for further work strains in which they did not appear. All the organisms studied by Winogradsky were physiologically similar to *Spirochaeta cytophaga*, being strictly adapted to cellulose and preferring nitrates or ammonia as a source of nitrogen. To the whole group he gave the generic name *Cytophaga*, rightly concluding that their morphology did not justify their inclusion in the genus *Spirochaeta*. On the basis of pigmentation and cell size five species were differentiated, of which the type species, *Cytophaga hutchinsoni*, was considered to be identical with *Spirochaeta cytophaga*.

Bokor (1930), claimed to have isolated a pure culture of *Spirochaeta cytophaga* on which he made morphological studies. As a result of these, he considered its cycle of development to be similar to that of the *Actinomycetes*, with which he placed it under the new name of *Mycococcus cytophagus*. The disparity between

Bokor's findings and those of all other workers in the field, as well as the illustrations in his paper, make it apparent that he was dealing with a mixed culture of *Spirochaeta cytophaga* and an *Actinomyces*.

In 1930 and 1933, Krzemieniewska subjected the previous work on the morphology of the cytophagas to a critical examination. She was able to show that Hutchinson and Clayton's *Spirochaeta cytophaga* and the organism described by Winogradsky as *Cytophaga hutchinsoni* were not identical, although very similar in vegetative morphology and pigmentation. In *Spirochaeta cytophaga* the coccus actually represented a stage in the developmental cycle, as had been claimed by Hutchinson and Clayton, whereas in *Cytophaga hutchinsoni* no such stage occurred. Her studies were facilitated by the discovery that the organisms could be grown on cellophane, and using this as a substrate she followed in detail the developmental cycle of *Spirochaeta cytophaga*, demonstrating beyond doubt the transition from rod to coccus and the subsequent germination of the coccus with the formation once more of the characteristic rod form. The whole process showed a remarkable similarity to the development of the myxobacteria belonging to the genus *Myxococcus*. In view of the lack of such a cycle in other cytophagas, Krzemieniewska was reluctant to place the whole group without reservation in the *Myxobacteriales*, and merely drew attention to the evidence in favor of a close relationship (1930):

Les recherches sur les espèces désignées par le nom de *Cytophaga*, sans aucun doute très proches à *Sp. cytophaga*. ne peuvent pas être considérés comme finis, il est donc possible qu'après quelque temps on pourra placer tout le groupe *Cytophaga*, y compris *Sp. cytophaga*, à côté des *Myxobacteriaceae* ou bien le compter parmi elles.

To the sporoids she gave the designation of microcysts, thus emphasizing their difference from the endospores of the *Eubacteriales*. *Spirochaeta cytophaga* she renamed *Cytophaga myxococcoides*.

In 1934, Stapp and Bortels described a number of new representatives of the genus which differed in pigmentation and tem-

perature optimum from the previously known species. One of these, *Cytophaga globulosa*, had a developmental cycle similar to that of *C. myxococcoides*, while the others did not form microcysts. It is possible that at least some of these forms are identical with previously described species.

An important discovery made by Stapp and Bortels was the existence of motility in the cytophagas. Although *a priori* this had seemed likely in view of the extreme regularity with which the organisms invested cellulose fibers, it had not been previously shown. It is true that Hutchinson and Clayton had observed rotatory and flexing movements in hanging drops, but Winogradsky could not confirm this. Winogradsky was also unable to demonstrate flagella, and suggested that movement might be brought about by very small haustoria. Stapp and Bortels observed a slow creeping movement in the direction of the long axis of the cell, quite different from the flagellar motility of the *Eubacteriales*, but were unable to substantiate Winogradsky's suggestion of the existence of haustoria. In addition to this they noticed flexing movements ("Krümmungsbewegungen"), but considered them to be pathological in nature.

In concluding this review, it is perhaps as well to point out some of the difficulties which have made work with the cytophagas so confused and contradictory. Due to their extreme physiological specificity, it is impossible to grow them in the absence of cellulose, which makes it extremely difficult to obtain pure cultures. Hutchinson and Clayton claim to have succeeded in this by the arduous and uncertain dilution method, but neither Winogradsky nor Krzemieniewska, the two principal workers in this field, had any of their forms in pure culture. Bokor's claims may be considered invalidated by his morphological findings. Stapp and Bortels were likewise unsuccessful except with their microcyst-forming species, *Cytophaga globulosa*. Their contention that the non-microcyst-forming species cannot be grown except in symbiosis with other forms has so far not been confirmed. A further obstacle, of particular gravity where morphological work is concerned, is the impossibility of growing the organisms in the absence of cellulose. This was partially obviated by Krze-

mieniewska's discovery that cellophane could be used as a substrate, but even on this medium, observation of the organisms in the living state is not easy, due to the fineness of the cells.

THE DISCOVERY OF TWO NEW CYTOPHAGAS

The above-mentioned difficulties do not exist in the case of some morphologically closely related bacteria which are, however, physiologically less specialized. They were encountered during the course of an investigation on marine agar-decomposing bacteria, and were easily obtained in pure culture. Two species have been differentiated: *Cytophaga krzemieniewskii*, n. sp.[1] and *Cytophaga diffluens* n. sp. Since the primary purpose of this paper is to draw attention to the morphology and type of motility of these forms, only their salient physiological and cultural characteristics will be mentioned here. Complete descriptions will be published later.

Both are strictly aerobic and grow well on a wide variety of media. Peptone and yeast extract are the only suitable nitrogen sources found, the organisms being unable to grow with inorganic nitrogen salts or any of a number of amino-acids in the presence of a suitable source of carbon and energy. Agar, cellulose, and some simpler carbohydrates, are decomposed, but starch and chitin are not attacked. Both strains liquefy gelatin and reduce nitrates, but do not produce indole or H_2S. Both are weakly catalase-positive. Growth on solid media is characteristically rapidly spreading; isolated colonies can never be obtained, the whole plate being covered with a thin layer of organisms in 2 to 3 days. *Cytophaga diffluens* produces a salmon pink pigment which slowly becomes orange on most media; *Cytophaga krzemieniewskii* produces a very pale pink pigment which is later masked by the production of a brown or black pigment.

The remarkable morphology of these organisms attracted attention from their first isolation. Both are large, gram-negative, non-sporeforming rods, occurring always in a variety of forms;

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[1] Named after Mme. H. Krzemieniewska, whose work on the soil cytophagas first indicated their true systematic position.

straight, arcuate, U-shaped, S-shaped, and even sometimes looped around into a full circle.

In young cultures, the cells of *C. diffluens* are 4 to 10 μ long with an average of about 7 μ, and 0.5 to 1.0 μ wide at the center. When examined in the living state, the ends are slightly pointed and the whole cell appears spindle-shaped, but in stained preparations these characters tend to disappear. As cultures become older the morphology changes; long threads twisted into bizarre shapes and often attaining a length of 20 to 30 μ occur. Star-shaped aggregates of cells are often formed in liquid media; this appears to be a process similar to the "Sternbildung" described by Stapp and Bortels for *C. globulosa.* No evidence of the production of spores or other resting stages has been found.

The cells of *C. krzemieniewskii* in young cultures are somewhat larger than those of *C. diffluens;* the length varies from 5 to 20 μ with an average of about 12 μ, and the width from 0.5 to 1.5 μ. Markedly spindle-shaped, pointed cells are rare; even in unstained preparations the majority are of more or less even thickness with slightly rounded ends. Star-shaped aggregates are commonly produced in liquid media. In older cultures the cells become swollen and refractile, but thread forms are never seen. Spores or other resting stages have never been found.

It is clear from the above account of the morphology of these two forms that they do not belong to any of the more common groups of rod-shaped bacteria. The only known simple rod-shaped bacteria which display these characteristics of extreme flexibility and spindle-shaped pointed cells are the cytophagas. Consequently, we were led to consider these two forms as representatives of this genus in spite of the fact that physiologically they differ from the previously known species.

MOTILITY OF THE CYTOPHAGAS

At first, all attempts to demonstrate motility failed. When the organisms were examined in wet mounts or hanging drops, no positive evidence of it could be observed, although occasionally rotatory movements or slight displacements of the cells were

noted. Although the organisms are strict aerobes, aerotaxis never occurred. Attempts to demonstrate flagella with *C. diffluens* failed, but in view of the difficulty of making satisfactory stained preparations of marine bacteria, this could not be regarded as conclusive. On the other hand, the extreme rapidity with which the organisms spread on solid media, only to be compared to the swarming of *Proteus vulgaris*, pointed to the existence of some method of locomotion. Since it seemed possible that movement might be restricted to a solid surface, another method of examination was adopted. A loopful of seawater-peptone-agar was streaked on a coverslip, allowed to solidify, and inoculated in several places with small masses of bacteria from the tip of a needle. The coverslip was then placed in position as the lid
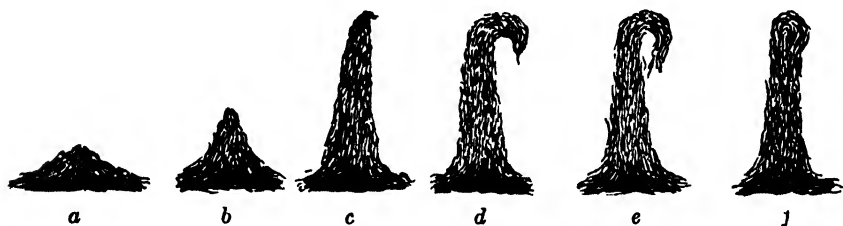
FIG. 1. CYTOPHAGA KRZEMIENIEWSKII. A TYPICAL MOVING MASS OF CELLS
a, concentration of cells at the periphery of the microcolony. *b–f*, subsequent stages in the formation and movement of the migratory mass. ×200.

of a moist chamber. After 15 to 60 minutes it was observed that bacteria had concentrated in small, pointed swellings at certain points along the peripheries of the "microcolonies" (fig. 1*a*). These presently began to move out across the agar (pl. 1, fig. 1). The moving masses, consisting of from 25 to more than a thousand cells, travel, each as a body, the majority of the individual cells being strictly oriented with their long axis in the direction of the movement. In the early stages, the mass is quite sharply pointed, a few cells at the front end acting as a spearhead. However, as movement continues, the shape may change in various ways. The head of the mass often loops back to join the main body of moving cells, forming a blunt, finger-shaped projection (fig. 1, *a–f*). Sometimes the tip turns around and rejoins the main colony, leaving a sterile island in the agar

surrounded by bacteria. More often, the migratory masses will meet, fuse, branch, and produce further outgrowths, ultimately leading to the formation of irregularly spaced fields of bacteria such as are shown in plate 1, figure 2.

The type of movement is completely different from the flagellar motility of the *Eubacteriales*. It consists of a slow, even gliding in one direction with none of the side-to-side movements or rapid directional changes associated with the motility of flagellated organisms. The rate of movement varies considerably, and is conditioned by at present unexplainable factors. Groups of cells in vigorous movement will suddenly slow up and stop, while others, previously immotile, will as suddenly start creeping across the agar. The movement of *C. diffluens* is in general much less rapid than that of *C. krzemieniewskii*. For the latter species, speeds of as much as 15 μ a minute have been noted, but the average is probably not much more than 5–6 μ a minute.

Particularly among moving cells, rapid flexing movements ("Krümmungsbewegungen") can be observed. These are sometimes caused by collision with some visible obstruction (such as a neighboring non-motile cell), but in other cases they seem to be purely spontaneous. They are evidently not causally related to the forward movement, since only a small minority of the cells in a moving mass exhibit them. As they occur in young, healthy cells under normal conditions of growth, it is unlikely that they are pathological in nature as was suggested by Stapp and Bortels (1934).

An interesting point is the apparent coordination between the different cells in a moving mass. While no physical connection between the cells is evident, the direction and speed of movement of a few cells at the apex control the activities of a large number of following cells. This is most clearly shown where an outgrowth consisting of perhaps a hundred cells has broken away from the microcolony and continued its movement across the agar in perfect order and coherence and at an undiminished rate. Single, isolated cells are very rarely motile, although occasionally they can be found separated by a distance of 10 μ or so from the nearest mass in a position which implies that independent move-

ment must have occurred. The unit of effective movement seems to consist of 20 to 30 cells.

SYSTEMATIC POSITION AND TAXONOMY

As already mentioned, the morphological similarities between the two species described here and the soil cytophagas are such that, in spite of the physiological differences which exist between them, we have been led to the conclusion that they are closely related. The demonstration of creeping motility and flexing movements in *C. globulosa*, a typical member of the soil group (Stapp and Bortels, 1934), is another evidence of the nearness of this relationship.

If we examine the physiological differences in more detail, it becomes clear that they are not as profound as would appear at first glance. Both *C. krzemieniewskii* and *C. diffluens* are able to decompose cellulose, although neither so rapidly nor so completely as the soil forms. The specialization of the latter group with regard to carbon and energy source as contrasted with the wider assimilatory powers of the former is an essentially secondary difference, and is found in many other groups of closely related bacteria, as for example the cellulose-decomposing vibrios. The differences in nitrogen requirements may also be more apparent than real. It is quite probable that *C. krzemieniewskii* and *C. diffluens* require certain growth factors, and that with these supplied they can use inorganic nitrogen just as well as peptone. There is an indication that even among the soil cytophagas special growth factor requirements may exist. The assertion of Stapp and Bortels that the non-microcyst-forming cytophagas studied by them were unable to grow except in symbiosis with other organisms can be explained logically by the assumption that these cytophagas are unable to synthesize certain growth factors which are supplied them by the symbionts.

The whole problem of the creation of genera on the basis of physiological specialization alone requires very careful consideration. For example, the genus *Cellvibrio*, created by Winogradsky for cellulose-decomposing vibrios growing "feebly" on "ordinary" media, presents considerable difficulties to the

taxonomist. Should organisms like *Vibrio agar-liquefaciens* and certain of the cellulose-decomposing vibrios described by Kalnins (1930) which may grow well on "ordinary" media even to the extent of preferring peptone to inorganic nitrogen, and which may utilize other energy sources much more rapidly and extensively than cellulose, be included in this genus? Where is the line to be drawn? For that matter, on the basis of the reasoning which led to the creation of the genus *Cellvibrio* a similar genus *Agarvibrio* might well be proposed, yet the only result would be further confusion.

More extensive research on the known cytophagas, and the discovery of new species may show the desirability of creating new genera within the group, but in the present highly incomplete state of our knowledge concerning them, Winogradsky's genus *Cytophaga* (although with a modified definition) may be used to cover all species with the exception of the two known to form microcysts. These clearly call for a separate position.

In considering the relationship of the cytophagas to the higher myxobacteria, we have two lines of evidence, which, taken in conjunction, make such a relationship certain. The first is the close analogy between the developmental cycles of the microcyst-forming cytophagas and of the myxobacteria belonging to the genus *Myxococcus*. Since this was conclusively demonstrated by Krzemieniewska (1930, 1933) no further discussion of it will be given here. The second is the similarity in the structure of the vegetative cell and the highly characteristic method of locomotion, which is exemplified most clearly for the cytophagas by *C. krzemieniewskii* and *C. diffluens*. The remarkable phenomenon of the production of fruiting bodies by the higher myxobacteria has tended to obscure the other less obvious, but even more important, characteristics of the group. An admirable description of these characteristics coupled with a recognition of their significance occurs in the following passage from Benecke's "Bau und Leben der Bakterien" (1912, p. 89):

Sind nun *alle* Bakterien in diesem weiteren Sinn derart gebaut, dass sie eine besondere Zellhaut besitzen? Dies scheint nicht der Fall zu sein. Man hat bei manchen Formen eine typische Zellhaut, wie sie der

echten Pflanzenzelle zu eigen ist, trotz grosser Sorgfalt nicht nach-
weisen können. Statt ihrer ist nur jene dichtere und festere Aussenlage
des Protoplasmas nachweisbar, von den Zoologen meistenteils als Pelli-
cula bezeichnet. . . . Es handelt sich hier hauptsächlich um die Zellen
der sog. Schleimbakterien, Myxobakterien, Formen die auch sonst
z. B. in ihrer Bewegungsweise, von Bakterien im engeren Sinn wesent-
lich abweichen . . . , ferner zumal auch dadurch dass ihre Zellen "flexil"
sind, d.h. die normalerweise gerade, stäbchenförmige Myxobakterien-
zelle kann sich kreisförmig biegen oder auch zusammenknicken, wobei
es zweifelhaft bleibt ob das Folge einer aktiven Krümmungsbewe-
gung ist.

These three characteristics of the vegetative myxobacterial
cell, namely, the absence of a rigid cell wall, the occurrence of
flexing movements and the peculiar method of locomotion, which,
as Benecke points out, are not found in the true bacteria, all
occur in the cytophagas. Even the rate of movement of 5 to 6
μ a minute established for *C. krzemieniewskii* compares closely
to that of 10 μ a minute given by Baur (1904) for the vegetative
myxobacterial cell. Furthermore, the pointed, spindlelike cell
shape so characteristic of the cytophagas is also found in many
of the myxobacteria (Jahn, 1924).

In view of this evidence, the absence of fruiting bodies can
hardly be considered a sufficient ground for the exclusion of the
cytophagas from the *Myxobacteriales*. Indeed, it would seem
more rational to characterize the order on the basis of the struc-
ture of the vegetative cell and the manner of locomotion, which
are sufficiently distinctive to exclude forms belonging to any
other order of bacteria, rather than on the basis of the production
of fruiting bodies and cysts. The cytophagas can then be in-
cluded as the simplest representatives of the order.

Once this is done, the further classification of the cytophagas
presents no difficulties. The microcyst-forming cytophagas can
be placed most conveniently in the family *Myxococcaceae*. The
absence of organized fruiting bodies prevents their being placed
in the genus *Myxococcus*, and calls for the formation of a new
genus. The following name and definition are proposed:

Genus **Sporocytophaga.** Spherical spores (microcysts) formed

loosely among the vegetative cells. Fruiting bodies absent. The type species is *Sporocytophaga myxococcoides*, syn. *Spirochaeta cytophaga* Hutchinson and Clayton and *Cytophaga myxococcoides* Krzemieniewska. *Not* synonymous with *Cytophaga hutchinsoni* Winogradsky.

It is further proposed to create a new family in the *Myxobacteriales* to include all those forms in which microcysts (spores) and fruiting bodies are absent, with the following name and definition:

Family **Cytophagaceae.** Long, flexible rods, often pointed and spindle-shaped. Creeping motility. No spores or fruiting bodies formed.

One genus, *Cytophaga* Winogradsky emend. Description as for family. The type species is *Cytophaga hutchinsoni* Winogradsky.

It is quite possible that simple myxobacteria of the *Sporocytophaga* and *Cytophaga* types have been observed and described in the past by workers who failed to realize their true taxonomic position. Such a failure would be understandable in the case of organisms not exhibiting the strict physiological adaptation to cellulose which has hitherto been considered so highly characteristic of the cytophagas, and which has been so strongly stressed, particularly by Winogradsky. Although a careful search of the literature has not been made, two papers have been encountered which are highly suggestive in this connection.

Johnson (1932) described briefly a series of chitin-decomposing bacteria, of which some were typical representatives of the genus *Myxococcus* while others failed to form fruiting bodies but resembled the myxococci in their vegetative morphology. Of the non-fruiting group some formed spores while others did not. Johnson considered it possible that the absence of fruiting bodies was due to the fact that a suitable medium for their production had not been used. However, in the light of our present knowledge, an equally logical explanation would be that these strains were actually representatives of the genera *Sporocytophaga* and *Cytophaga*. This is a point which can be settled only by long-continued examination of the organisms in question on a variety of media.

Benton (1935) has also described certain chitin-decomposing bacteria which might conceivably belong to the cytophagas.

Unfortunately, cultures of the organisms obtained by these workers are no longer available, so that for the present the question of their position must remain an open one. Nevertheless, it does not seem unlikely that the simple myxobacteria are quite a widespread group. Henrici, in a private communication, has also suggested this:

I suspect that there is a large group of bacteria similar to the *Myxobacteria* but failing to produce definite fruiting bodies. This, however, may be due to a failure to find an appropriate medium for fruiting.

PHYLOGENY OF THE MYXOBACTERIALES

In view of the above outlined thesis that the cytophagas are primitive representatives of the *Myxobacteriales*, it becomes of interest to speculate on the phylogeny of this order.

In the first place, no support can be provided for the hypothesis that a close relationship exists between the *Myxobacteriales* and the *Eubacteriales*. The fact that the vegetative cells of these two orders are analogous and not homologous in structure is well brought out by the cytophagas, although it was already quite evident from a consideration of the higher myxobacteria alone (Benecke, 1912, Jahn, 1924).

A much more plausible hypothesis in the light of present knowledge is the derivation of the myxobacteria from the *Myxophyceae*, which was suggested by Jahn in 1924. Among the unicellular blue-green algae there are several little-known genera whose morphological resemblances to the cytophagas are striking. This is particularly true of certain species of the genera *Rhabdoderma* and *Dactylococcopsis*, in which spindle-shaped, pointed cells, often arcuate or S-shaped, are found. Furthermore, the highly characteristic creeping motility of the myxobacteria also occurs in the blue-green algae. While commonly associated with the more complex representatives of this class, such as *Oscillatoria*, it has been shown by Jahn (1924) to exist also among the unicellular forms. The production of slime, probably connected with the mechanism of movement (see Jahn, 1924), is another common characteristic of the two groups.

Two other hypotheses concerning the relationships of the myxobacteria have been proposed, but there is no substantial evidence in support of either. The possibility of a relationship to the myxomycetes, tentatively suggested by Thaxter (1904) and strongly upheld by Vahle (1910) has been adequately dealt with by Jahn (1924). Thaxter's (1904) suggestion of a relationship with the sulfur bacteria can also be discounted. It was based on the hypothesis that the pink color often shown by myxobacterial cells in the mass was due to the presence of a pigment similar to bacteriopurpurin. This would imply the existence of a photosynthetic mechanism in the myxobacteria, for which there is no evidence. Furthermore, the purple bacteria resemble the *Eubacteriales* very closely morphologically, so that the objections outlined above to a connection of the myxobacteria with the true bacteria hold equally for the purple bacteria. Buchanan (1939), in his general discussion of the myxobacteria which was prepared for the fifth edition of Bergey's Manual, makes the statement:

The *Myxobacteriales* may be regarded as a well differentiated order of the *Schizomycetes* resembling the true bacteria on the one hand and the *Myxophyceae* (*Cyanophyceae*) and *Thiobacteriales* on the other.

However, it is probable that Buchanan considers primarily the family of the *Beggiatoaceae* among the *Thiobacteriales* as relatives of the *Myxobacteriales*. The *Beggiatoaceae* are, however, not related to the purple sulfur bacteria and the *Athiorhodaceae* (although still placed in the same order in Bergey's Manual), but may be considered to be colorless representatives of the filamentous blue-green algae. In view of this it is not surprising that they show certain resemblances to the myxobacteria, if we consider the latter group as also having developed from the blue-green algae. However, the *Beggiatoaceae* and the myxobacteria probably had their origins in widely separated groups of the *Myxophyceae*; the similarities between them are very slight.

Summing up on the basis of the existing evidence, the most plausible hypothesis is that the *Myxobacteriales* are an end group in evolution, derived originally from unicellular *Myxophyceae*. They have diverged from these through the loss of the photosyn-

thetic mechanism (with consequent adaptation to a saprophytic existence) and the gradual development of highly organized fruiting bodies.

SUMMARY

Two new representatives of the cytophagas have been isolated which differ in certain respects from the previously known members of this group. A detailed study of their morphology and method of locomotion has been made. On the basis of this and previous work, it is proposed that the cytophagas be included in the *Myxobacteriales* as the simplest representatives of this order. It is proposed to create a new genus *Sporocytophaga* in the family *Myxococcaceae* for those cytophagas which produce spores (microcysts), and a new family *Cytophagaceae* with one genus, *Cytophaga* Winogradsky emend., for the non-spore-producing forms. The phylogeny of the *Myxobacteriales* is discussed in the light of these new concepts.

ADDENDUM

While the manuscript of this paper was in press, the writer's attention was drawn to two publications by Imsenecki and Solntzeva (Bull. Acad. Sci. URSS, 1936, pp. 1115–1172; Mikrobiologia, 1937, v. 6, pp. 3–15) in which essentially the same ideas on the systematic position of the cytophagas have been advanced.

In conclusion, the writer wishes to express his gratitude to Prof. C. B. van Niel for much criticism and advice.

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PLATE 1

FIG. 1. *Cytophaga krzemieniewskii*. Edge of a microcolony, showing several masses of cells in the early stages of movement. ×75.

FIG. 2. *Cytophaga krzemieniewskii*. Edge of a microcolony several hours older than that shown in figure 1. The irregularly scattered fields of bacteria are formed by the fusion of separate masses, followed by further migration. ×75.

FIGS. 3–5. *Cytophaga krzemieniewskii*. Successive stages in the development of a moving mass, taken during a period of 10 mins. ×340.

All figures drawn from photomicrographs.

(R. Y. Stanier: Studies on the Cytophagas)

CULTURE MEDIA FOR BRUCELLA

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Of the media now available for the cultivation of *Brucella*, none is very satisfactory. With liver infusion agar we have observed many times that if a given inoculum will yield 600 or 650 colonies on a poured plate, an inoculum half as large will yield absolutely no colonies. Bacto-Tryptose medium, recently introduced by the Difco Laboratories, Detroit, seems to give a quicker initial growth, and is improved by the addition of nicotinic acid and thiamin chloride, as reported in a previous paper (Kerby, 1938). It is apparent, however, that if a medium is to be reliable for clinical use, it must be much better than those now available. Opportunity to attempt solution of this problem was afforded through support of the Clayton Foundation for Research, with the following results.

A large number of materials, including various vitamins, were investigated for their effect as growth-promoting factors for *Brucella* when added to the basic medium, Bacto-Tryptose broth. In the absence of a known method for insuring absolutely uniform inoculation of every series of media, ratings have been made on the basis of a standard comparison of each series with tryptose broth, as prepared in uniform lots by Difco. Since the desired medium would be one yielding growth from a small inoculum, such an inoculum was sought in every instance. For each series, the inoculum consisted of a uniform amount (usually 0.05 or 0.1 ml.) of a dilution (usually 1:400) of the following: 5 ml. of physiologic saline to which was added one flat loopful (2 mm.) of a light broth suspension of *Brucella*. Each successive dilution was mixed vigorously and uniformly before transferring to the next tube, in arriving at the final, desired dilution. In 61 series, this

procedure resulted in an average inoculum of 108 viable organisms, as checked by tryptose agar plate counts, with extremes ranging from less than 1 (no growth on inoculum control plate, but *Brucella* recovered from the tryptose broth control or the test medium or both) to 322. Media were tubed in 15 ml. amounts. After inoculation and incubation of each series, growth was observed by daily subculture. In transplanting, each tube was again vigorously and uniformly mixed, and 0.1 ml. transferred to tryptose agar for a poured plate. These plates were incubated uniformly for maximal growth, and plate counts recorded. The daily subcultures were continued until innumerable colonies were obtained on the plates from the media or until definite inhibition was established. Where an old stock culture was employed in the tests, 3 to 5 days' incubation usually sufficed to establish a comparative record. Longest incubation was 28 days. Repeat tests were run, in duplicate, wherever results warranted. The following summary includes unfavorable as well as favorable results, in the hope of saving others from the time-consuming task of ruling out certain substances possessing possible value.

Milk medium containing 2 per cent Bacto-Tryptose, 0.5 per cent sodium chloride, 1:700,000 crystal violet, made up with fresh, whole, Grade A pasteurized milk and sterilized by tyndallization, showed earlier and more abundant growth than that obtained in a tryptose broth control medium. Also, growth was positive in the milk medium when the inoculum had been reduced beyond the point where *Brucella* could be recovered from tryptose broth (tables 1 and 2). The use of skimmed milk, homogenized milk, powdered milk, certified raw milk, or heavy cream as a base in this medium was not satisfactory. With skimmed milk, growth was only slightly better than in tryptose broth. Homogenized milk medium was somewhat less effective than whole milk medium, while both certified raw milk and heavy cream media appeared to have an inhibitory effect on growth of *Brucella*. A similar effect was observed with the powdered milk media. Incorporation of a 50 per cent liver-infusion base into the milk medium seemed to have a slightly favorable effect, but the results were not convincing. Media made with peptonized or

trypsinized milk base were inferior to tryptose broth. Further
investigation revealed, moreover, that the whole pasteurized

TABLE 1

INOCULUM OF 0.05 ML. SUSPENSION (PLATE COUNT = NO GROWTH)	GROWTH ON SUBCULTURE OF 0.1 ML. TO TRYPTOSE AGAR POUR PLATES			
	First day	Second day	Third day	Fourth day
	colonies	*colonies*	*colonies*	*colonies*
Milk medium.......... ...	0	118	Innumerable	Innumerable
Tryptose broth	0	0	0	0
INOCULUM OF 0.025 ML. SUSPENSION (PLATE COUNT = NO GROWTH)				
Milk medium	0	40	2200	Innumerable
Tryptose broth.	0	0	0	0

Suspension of *Brucella abortus* 456 was used. 0.05 ml. also plated out to de-
termine size of inoculum by plate count.

TABLE 2

INOCULUM OF 0.1 ML. SUSPENSION (PLATE COUNT = 55 COLONIES)	GROWTH ON SUBCULTURE OF 0.1 ML. TO TRYPTOSE AGAR POUR PLATES					
	First day	Second day	Third day	Fifth day	Sixth day	Twelfth day
	colonies	*colonies*	*colonies*	*colonies*	*colonies*	*colonies*
Milk medium ..	0	1	88	Innumerable	Innumerable	
Tryptose broth..	0	0	13	About 12000	Innumerable	
INOCULUM OF 0.1 ML. SUSPENSION (PLATE COUNT = 40 COLONIES)						
Milk medium ..	0	0	19	Innumerable	Innumerable	
Tryptose broth..	0	1	17	About 800	Innumerable	
INOCULUM OF 0.1 ML. SUSPENSION (PLATE COUNT = 4 COLONIES)						
Milk medium. ..	0	0	0	0	0	Innumerable
Tryptose broth..	0	0	0	0	0	0

Suspension of *Brucella abortus* 456 used, diluted to obtain various inocula.
0.1 ml. of each dilution also plated out to determine size of inoculum by plate
count.

milk medium was not dependable, variation in the lot of milk used
causing great variation in the efficiency of the medium. Some

lots were much superior to tryptose broth (tables 1 and 2); other lots were encountered which were no better than or decidedly inferior to tryptose broth in their ability to support growth of *Brucella* (table 3). Obviously a method of standardizing the medium must be developed before it can be used in routine work.

TABLE 3

Suspension of Brucella abortus 456 used

0.05 ml. also plated out to determine size of inoculum by plate count in each of these studies.   x = subcultured on tryptose agar slants only.

INOCULUM OF 0.05 ML. SUSPENSION (PLATE COUNT = 57 COLONIES)	GROWTH ON 0.1 ML. SUBCULTURE TO TRYPTOSE AGAR POUR PLATES			
	First day	Second day	Third day	Fourth day
	colonies	*colonies*	*colonies*	*colonies*
Sodium caseinate medium...	5	1007	Innumerable	Innumerable
Milk medium*	1	27	About 2000	Innumerable
0.1% tryptose agar.	2	56	About 2600	Innumerable
Tryptose broth.............	2	65	About 2550	Innumerable
INOCULUM OF 0.05 ML. SUSPENSION (PLATE COUNT = 33 COLONIES)				
Sodium caseinate medium ..	x	1934	x	x
Milk medium*..............	x	9	x	x
0.1% tryptose agar.........	x	0	x	x
Tryptose broth.............	x	246	x	x
INOCULUM OF 0.05 ML. SUSPENSION (PLATE COUNT = 75 COLONIES)				
Sodium caseinate medium...	x	3013	x	x
0.1% tryptose agar.......	x	11	x	x
Tryptose broth.............	x	475	x	x

* It will be noted that this lot of milk medium, although prepared by the standard method adopted for study, is in no way superior to the tryptose media controls.   Compare with results shown in tables 1 and 2.

Tryptose agar made up with a milk base proved a poor medium for *Brucella*.

McNutt and Purwin (1930) described a nutrose (sodium caseinate) carbohydrate medium which they found capable of supporting growth of *Brucella*. Various sodium caseinate media were studied in the present series, and the following one appeared most effective in growing *Brucella abortus* 456: 2 per cent sodium case-

inate, 2 per cent Bacto Tryptose, 0.1 per cent Bacto agar, 0.5 per cent sodium chloride, 1:700,000 crystal violet. The advantage of a small quantity of agar in liquid medium has been observed frequently (Hitchens, 1921; Zobell and Meyer, 1932; Meyer and Zobell, 1932). When prepared with a milk rather than distilled water base, the medium proved wholly unsatisfactory. Preparation of sodium caseinate medium can be expected to result in uniform lots of media, but the medium does not appear to be uniformly good for all strains of *Brucella*. Of the series of organisms tested, the growth of *Brucella abortus* 456 only was enhanced. Eleven other stock strains failed to show improved growth in sodium caseinate medium over that in the tryptose broth controls. The series included 1 recently isolated *Brucella melitensis* strain, 4 old *B. melitensis* strains, 1 old porcine strain, 2 recently isolated *B. abortus* strains and 4 old *B. abortus* strains. The value of sodium caseinate medium in routine culture work is therefore questionable.

Addition of cod liver oil (16 per cent) to milk media resulted in inhibition of growth. The same result, complete or partial, was obtained when cod liver oil (16 per cent) or cod liver oil in Maltine (16 per cent) was incorporated into tryptose broth medium. Various tomato juice media (50 to 100 per cent) failed to support growth of the organism. Addition of carrot extracts (7, 35, 70 per cent) to tryptose broth resulted in slight inhibition of growth. Attempts to enrich tryptose media by addition of vitamins A, B_1 and B_2 in the form of Embo products were unsuccessful, Embo proving inhibitory. The same result was obtained upon addition of Hormodin A, a substance said to stimulate growth of plant roots, (1:50 through 1:1,000,000) and of anterior pituitary extract (Growth-Promoting Hormone, Squibb) in varying concentrations (1:100 through 1:10,000). Many variations of a basic spleen-infusion agar were investigated. None proved superior to tryptose agar. Addition of glutathione (10 to 40 mgm. per liter) to tryptose media resulted in no significant improvement of the media, in the concentrations tried. Yeast extract (0.3, 0.5, 1.0 per cent) likewise had little effect (Hutner, 1938; Wood, Anderson, and Werkman, 1938). A slightly inhibitory effect was

noted in media containing Bacto Asparagine (0.5, 2.0 per cent), Bacto Malt Extract (2, 10 per cent), bone meal extracts, bone marrow extracts, purified corn phosphatid (0.5, 2.0 per cent) and soy bean extracts (4, 10 per cent), respectively. Tryptose medium was not improved by the addition of *Bacillus subtilis* extracts (Zobell and Meyer, 1932), nor by preparation with a gelatin (10 per cent) or whey (3 per cent) base. Sodium sulfite, 0.05 per cent, in tryptose broth, (Wright, 1934) exerted a slightly inhibitory influence on *Brucella*.

Liver-infusion medium, prepared according to the method of Hasley and Schalter (1939) was not as satisfactory in our hands for the cultivation of *Brucella* as is tryptose broth. The same was true of the Burky modification of Huntoon's hormone medium (Huntoon, 1918). Brewer's sodium thioglycollate medium (Brewer, 1940) also failed to support adequately the growth of *Brucella*, whether prepared with a pork infusion or a tryptose base.

SUMMARY

Of numerous substances studied for their possible value as growth-promoting factors for *Brucella*, only two seem worthy of further investigation. A milk medium described may prove useful if variation due to undefined differences in lots of milk can be controlled. Sodium caseinate medium seems to show promise in the isolation of certain strains of *Brucella*, but it probably cannot be relied upon to improve tryptose broth in all instances.

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* References of general interest, but not quoted directly in the text.

THE USE OF DEHYDRATED THIOGLYCOLLATE MEDIUM IN THE ENRICHMENT OF SPORE-FORMING ANAEROBIC BACTERIA

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An important advance in the technique of culturing the anaerobic bacteria is reflected in the report of Brewer (1940) who suggests a clear fluid medium in which sodium thioglycollate is the reducing agent.   Before such a medium can be accepted for the enrichment cultivation of anaerobic bacteria from sutures, surgical specimens or other materials it is necessary to show that growth may be initiated from relatively small numbers of vegetative cells or spores.   With this in mind a series of comparative tests have been made using several strains each of the following clostridia: *Clostridium welchii, C. oedematiens, C. septicum, C. oedematoides, C. tetani, C. parabotulinum, C. sporogenes, C. acetobutylicum, C. felsineum, C. roseum* and *C. butyricum.*   Thus a variety of physiological types were tested.

The media used included:

1. Dehydrated thioglycollate medium.[2]   This medium is prepared from pork infusion and the samples tested contained 1.0 per cent glucose and methylene blue (1:500,000).

2. Liver infusion broth with liver particles prepared according to the formula of McClung and McCoy (1934).

3. Liver infusion broth (as above) with 0.1 per cent sodium thioglycollate[2] and 0.01 per cent agar.

4. Beef-heart infusion with meat particles: Steep overnight in ice box 400 grams of fat-free minced beef heart in 1 liter of cold tap water. Add 10 grams bactopeptone and 5 grams table salt (NaCl).   Boil over

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[1] Fellow of the John Simon Guggenheim Memorial Foundation.

[2] Supplied by Baltimore Biological Laboratories, Baltimore, Maryland.

free flame 10–15 minutes and strain through wire sieve.   Add 22 ml. of N/1 NaOH per liter to broth and boil 30 minutes over free flame.

TABLE 1

Typical protocol showing relative value of various anaerobic media

	DILUTION OF INOCULUM	DEHYDRATED THIOGLYCOLLATE MEDIUM	LIVER INFUSION WITH PARTICLES	LIVER INFUSION BROTH PLUS THIOGLYCOLLATE	BEEF HEART INFUSION WITH PARTICLES	BEEF INFUSION BROTH PLUS THIOGLYCOLLATE	CORN-LIVER MEDIUM
C. oedematiens	10^{-3}	+	+	+	+	+	+
	10^{-4}	+	+	+	+	+	+
	10^{-5}	+	+	+	+ or −	−	−
	10^{-6}	−	+	−	+ or −	−	−
	10^{-7}	−	−	−	−	−	−
	10^{-8}	−	−	−	−	−	−
C. welchii	10^{-5}	+	+	+	+	+	+
	10^{-6}	+	+	+	+	+	+
	10^{-7}	+	+	+	+	−	−
	10^{-8}	−	−	−	−	−	−
	10^{-9}	−	−	−	−	−	−
C. septicum	10^{-5}	+	+	+	+	+	+
	10^{-6}	+	+	+	+	+ or −	−
	10^{-7}	−	−	−	−	−	−
	10^{-8}	−	−	−	−	−	−
C. tetani	10^{-5}	+	+	+	+	+	+
	10^{-6}	+	+	+	+	+	−
	10^{-7}	+ or −	+	+ or −	−	−	−
	10^{-8}	−	−	−			
C. sporogenes	10^{-5}	+	+	+	+	+	+
	10^{-6}	+	+	+	+	+	+
	10^{-7}	−	+	+ or −	−	−	−
	10^{-8}	−	−	−			
C. acetobutylicum	10^{-4}	+	+	+	+	−	+
	10^{-5}	−	+	+	−	−	+
	10^{-6}	−	+	−	−	−	+
	10^{-7}	−	−	−	−	−	−
	10^{-8}	−	−	−	−	−	−

Filter hot through paper and tube broth over meat particles saved from above.   Autoclave 2 hours at 15 pounds pressure.

5. Beef infusion broth (as above) with 0.1 per cent sodium thioglycollate and 0.01 per cent agar.

6. Corn-liver medium of McClung and McCoy (1934).

Two or more tubes of each medium were inoculated with 1.0 ml. of decimal dilutions (from 10^{-3} to 10^{-9}) of active broth cultures of each of the strains tested. All tubes were inoculated (without reheating) approximately 15 hours after sterilization. The temperature of incubation was 37°C.

<div align="center">RESULTS</div>

Space will not permit the inclusion of all of the protocols for these tests but the results may be given in general terms. Typical protocols are reproduced in table 1. With the pathogenic forms (and *C. sporogenes*) the best media (i.e., those which initiated growth from highest dilution of inoculum) were the dehydrated thioglycollate medium and the liver infusion with liver particles. With the beef heart medium with meat particles and with the liver broth plus thioglycollate the results were almost as good except that with an occasional strain growth did not occur in the highest dilution positive with the first mentioned media. With the beef infusion broth plus thioglycollate occasional negative tubes in high dilutions were encountered. With the butyric acid and butyl alcohol group the dehydrated thioglycollate medium was not as satisfactory as the others; with these organisms the liver infusion with liver particles and the corn-liver medium were the best.

<div align="center">SUMMARY AND COMMENT</div>

Since the dehydrated thioglycollate medium of Brewer (1940) compares favorably with the complex meat infusions in the ability to initiate growth from small numbers of cell, it is suggested that this medium be given extensive trial, at least as an auxiliary medium, in attempts at the enrichment of the pathogenic and proteolytic anaerobic bacteria. The ease of preparation and the fact that the medium stays reduced for a long period (in days) after sterilization, permitting inoculation without reheating, would

argue for its general adoption. For the butyric acid and butyl alcohol group the corn-liver medium (McClung and McCoy, 1934) is to be preferred. In view of the results obtained with liver infusion broth plus thioglycollate, it is suggested that a dehydrated medium with a liver base may prove even more acceptable than the present medium in which the base is a pork infusion.

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THE pH REQUIREMENTS OF SOME HETEROFERMEN-
TATIVE SPECIES OF LACTOBACILLUS

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I. INTRODUCTION

The lactobacilli are noted for their ability to grow in acid media. Although media with reactions between pH 3.5 and 5.0 have been recommended for their isolation, the media used in physiological studies by Pederson (1929, 1938), Nelson and Werkman (1935, 1936), Weinstein and Rettger (1932) and others had values ranging from pH 6.2 to 7.0.

Shimwell (1935) found the optimum pH for the growth of *Lactobacillus pastorianus* to be approximately 8.0. It has been observed however that species of *Lactobacillus* isolated from wines and other similar products thrive best when the medium is appreciably acid. Otani (1936) pointed out that homofermentative lactobacilli isolated from sake grew most vigorously between pH 4.0 and 5.0 Fornachon (1936) found the optimum pH for the growth of a heterofermentative species of *Lactobacillus* isolated from Australian wine to be between pH 4.0 and 5.0. Douglas and McClung (1937) reported a bacterium from California wine with an optimum pH between 4.1 and 4.3. (This organism is a heterofermentative species of *Lactobacillus* according to unpublished data of Douglas and Vaughn.) Arena (1936) described several lactic acid bacteria isolated from Argentinian wine and found the optimum pH for growth to vary between pH 4.3 and 6.7.

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Morishita (1929) working with aciduric organisms from dental caries found the best growth between pH 5.0 and 6.8. Longworth and MacInnes (1935) reported the greatest acid production by *Lactobacillus acidophilus* at pH 5.5 to 6.0. Weiss and Rettger (1934) determined the pH range for best growth of *Lactobacillus bifidus* as 5.4 to 6.5, and for *Lactobacillus acidophilus* as 5.8 to 6.4.

In the investigations cited above the media were not all the same. It is known that the nature of buffers and other constituents, as well as the individual characteristics of the bacteria studied, may influence the response to pH and it is not surprising that variations have appeared.

The following study was undertaken to determine more closely the pH range for maximum growth and metabolism of certain gas-forming lactobacilli, and to point out the effect of pH on growth and fermentation reactions of these bacteria.

II. THE ORGANISMS

The bacteria used included three cultures of *Lactobacillus brevis* and four cultures of *Lactobacillus hilgardii* isolated from California wines together with the following species: *Lactobacillus fructovorans* and *Lactobacillus gracilis* from Dr. C. S. Pederson and *Lactobacillus pentoaceticus* from the University of Wisconsin collection.[2]

III. EXPERIMENTAL

a. Optimum pH for growth

Cell counts were used in determining the optimum pH for cell multiplication. Preliminary experiments having shown the optimum pH to be approximately 5.5, equal quantities of the test organisms were sown in a series of flasks of yeast water medium containing 2.0 per cent glucose. The pH was adjusted to approximately 4.5, 5.5 and 7.0 with M/4 phosphate-acetate buffers and measured before inoculation and after completion of

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[2] *L. pentoaceticus* is now considered a synonym for *Lactobacillus brevis*. The exact taxonomic position of *L. fructovorans*, *L. hilgardii* and *L. gracilis* is doubtful. See Pederson in Bergey's Manual of Determinative Bacteriology, 5th edition, 1939. *Lactobacillus hilgardii* has been redescribed by the authors and the results will be published.

the experiment with the quinhydrone electrode. The culture flasks were incubated at 30°C. Cell counts were made at intervals by use of a Petroff-Hausser bacteria counter. Typical growth curves are shown in figure 1.

The beneficial effect of an acid reaction is shown. More cells were produced at pH 4.65 and 5.45 than at pH 7.0.

b. *Optimum pH for dissimilation of glucose*

The optimum pH for the dissimilation of glucose was determined with suspensions of cells which were prepared as follows: Ten liters of yeast water containing 2.0 per cent glucose were inoculated with one liter of a 24-hour culture grown in glucose
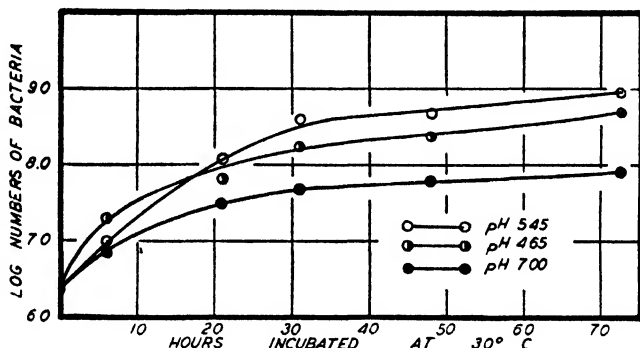
FIG. 1. EFFECT OF pH ON GROWTH OF LACTOBACILLUS HILGARDII No. 5

yeast water. After 24 hours incubation at 30°C, the bacteria were separated from the culture aseptically by means of a Sharples super-centrifuge and suspended in 250 ml. of 0.9 per cent NaCl solution.

Ten ml. of this cell suspension were placed in each of a series of large sterile tubes containing 2.5 ml. of sterile 20.0 per cent glucose solution, 5.0 ml. of sterile 0.9 per cent NaCl solutions and 7.5 ml. of sterile phosphate-acetate buffer of the desired pH. The buffer solutions were so prepared that the final dilutions in the tubes were approximately M/4 with respect to both phosphate and acetate. The number of cells in the mixture was approximately 2×10^{10} per cubic centimeter. Under these conditions a temperature of 37°C. was best for the dissimilation of glucose

and all experiments with cell suspensions were conducted at this temperature.

The glucose in the fermenting liquid was determined after various intervals of time by the ceric sulfate method as described by Hassid (1936, 1937) and results expressed in terms of grams of glucose decomposed per 100 ml.

Table 1 shows the rates of glucose dissimilation by suspensions of *L. hilgardii* strain 7. Under the conditions of this experiment the optimum pH for glucose destruction was about 5.5.

TABLE 1

Effect of pH on dissimilation of glucose by suspensions of Lactobacillus hilgardii culture 7

TUBE NUMBER	INITIAL pH	GLUCOSE* UTILIZED IN GRAMS/100 ML. AFTER				FINAL pH
		2 hours†	4 hours	6.5 hours	8 hours	
1	4.05		0.040	0.115	0.145	3.92
2	4.46	0.200	0.335	0.460	0.558	4.28
3	4.96	0.280	0.615	0.860	1.095	4.58
4	5.46	0.480	1.022	1.334	1.475	4.88
5	5.96	0.250	0.750	1.048	1.238	5.29
6	6.43	0.420	0.667	0.910	1.045	5.94
7	6.86	0.370	0.680	0.904	0.960	6.44
8	7.60	0.200	0.380	0.528	0.550	7.07

' Initial concentration of glucose 2 grams per 100 ml.
† Incubation temperature 37°C.

c. Optimum pH for decomposition of l-malic acid

Table 2 shows the effect of pH on the decomposition of l-malic acid by growing cultures. None of the organisms attacked the acid at pH 7.0. The optimum pH range appeared to be between 4.0 and 5.5.

d. Effect of pH on the utilization of sugars

Table 3 shows the effect of pH on acid production from various sugars important for taxonomic purposes.

The medium was yeast water containing 1.0 per cent of test carbohydrate. The basal medium and 10.0 per cent sugar solutions were sterilized separately and the sugar added aseptically

TABLE 2

Effect of pH on decomposition of l-malic acid

ORGANISM[*]	DECREASE IN TITRATABLE ACIDITY EXPRESSED AS ML. N/10 BASE/10 ML.								
	pH								
	4.0	4.5	5.0	5.5	6.0	6.5	7.0	7.5	8.0
L. gracilis	2.3	2.6	2.3	1.7	0.3	†			
L. pentoaceticus	2.6	3.4	3.0	2.9	1.6	0.7			
L. fructovorans.....			0.4	0.3					
L. brevis‡..........		0.1	0.4	0.3					
L. hilgardii§.......			0.5	0.3					
L. hilgardii§......	2.5	2.6	2.6	1.8	0.4	0.2			

* Incubated for 35 days at 30°C.

† Blank indicates no change in titratable acidity.

‡ Figures represent average of three cultures.

§ Figures represent average of two cultures.

The medium: 1.0 per cent Bacto-tryptone, 0.5 per cent Bacto yeast extract, 0.1 per cent K_2HPO_4, 0.5 per cent l-malic acid dissolved in distilled water. Sterilized by filtration through Berkfeld candle.

TABLE 3

Effect of pH on utilization of various sugars

Increase in titratable acidity as ml. N/10 base/10 ml.

SUGAR[*]	L-ARABINOSE		D-XYLOSE		GLUCOSE		FRUCTOSE	
	pH							
	6.8	5.5	6.8	5.5	6.8	5.5	6.8	5.5
L. pentoaceticus.... ..	11.70	10.00	11.74	10.90	2.70	2.60	6.20	4.64
L. fructovorans	†			0.30		5.24	5.00	4.40
L. brevis‡	9.10	8.50	12 90	11.10		4.54	4.62	2.92
L. hilgardii‡..........			7.70	11.64		7.12	5.90	6.00
L. hilgardii‡..........			9.00	8.60		4.42	4.80	4.64

SUGAR	GALACTOSE		LACTOSE		MALTOSE		SUCROSE	
L. pentoaceticus	4.20	2.50			5.40	2.90	1.10	0.90
L. fructovorans								
L. brevis......		4.00		2.70		4.44		2.50
L. hilgardii..........		5.70		1.00		5.60		7.20
L. hilaardii...		4.40		0.90		4.80		3.00

* Incubated for 14 days at 30°C.

† Blank indicates no change in titratable acidity.

‡ Figures represent average of several cultures. *L. hilgardii* cultures divided on basis of decomposition of l-malic acid as in table 2.

Correction was made for acid production in control cultures containing no sugar.

to the basal medium.   Inoculations consisted of three standard loops (4 mm.) of young liver-infusion broth cultures.

The effect of pH is particularly striking with respect to the utilization of glucose and certain of the disaccharides.   Glucose, galactose, lactose, maltose and sucrose were actively fermented in yeast water at pH 5.5.   At pH 6.8 these sugars were not attacked.   Similar results were obtained in tryptone yeast-extract medium.

An experiment to test the possible chemical inversion of the disaccharides at pH 5.5 showed no inversion of lactose, maltose or sucrose, in the yeast juice medium after 7 days at 30°C.   All of the cultures showing acid production for these sugars at pH 5.5 were growing 3 days after inoculation.   Furthermore, if inversion occurred at pH 5.5 it would be expected that *L. fructovorans* and *L. pentoaceticus* would show acid production from all of the disaccharides.

IV. DISCUSSION AND CONCLUSIONS

The optimum pH for growth did not differ greatly from that for metabolism of carbon compounds for the organisms investigated, both being greatest in the range of pH 5.0 to 6.0.   This fact may be of little taxonomic importance so far as the vigorously growing species are concerned, but it should be given due consideration when carrying out studies on those cultures more exacting in their requirements for growth and metabolism; under such conditions the determination of optimum pH is quite as important as the determination of optimum temperature range.

When the gas-forming lactobacilli are grown in unbuffered media containing carbohydrates which are readily attacked, the adverse effect of a neutral reaction is quickly obscured, but with carbon sources less readily decomposed or whose decomposition is not accompanied by acid production causing a fall in pH, (as in the case of malic acid) the pH of the medium may be the limiting factor in determining whether they are decomposed.   Attention should be given to the optimum pH for growth and metabolism when investigating the heterofermentative species of *Lactobacillus*.

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A MOTOR-DRIVEN, MECHANICALLY SUPPORTED ULTRACENTRIFUGE FOR THE SEPARATION OF BIOLOGICAL MATERIALS[1]

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The use of intense centrifugal fields of from 50,000 to 100,000 times gravity, or greater, has become a technic of the first importance for the separation of biological materials. Various types of quantity-ultracentrifuges have been devised for this kind of service, most of them embodying an adaptation of the principle of Henriot and Huguenard (1925) according to which the rotating member is supported and driven by compressed air. Such centrifuges have been applied most extensively to the purification of viruses (Stanley, 1938), although results have been reported which indicate that they may be useful in the separation of substances of smaller unit size, such as antibodies (Wyckoff, 1936; Heidelberger and Pedersen, 1937; Gratia and Goreczky, 1938) and hormones (Severinghaus, Levin and Chiles, 1938). The scope of these centrifuges is in fact limited only by the structural strength of their rotors. The limits for safe routine operation have been defined by Wyckoff and Lagsdin (1937) as 45,000 r.p.m. for the quantity heads of Dowmetal and 60,000 r.p.m. for those of duralumin 14ST, 6½ to 7 inches in diameter, which have been

commonly employed in the air-driven ultracentrifuge. These speeds corresponded to a maximal tube-field of 180,000 times gravity for the heads of Dowmetal and 320,000 for those of duralumin 14ST. These limits suffice, however, for a variety of biological problems. For example, one of the smallest viruses, that of poliomyelitis, may be completely sedimented within a few hours in a tube-field of from 50,000 to 100,000 times gravity (Schultz and Raffel, 1937).

The present communication describes a motor-driven, mechanically supported quantity-ultracentrifuge having a maximal speed of 44,000 r.p.m., producing a maximal tube-field of 150,000 times gravity in the rotor which was employed. It is believed that the design affords advantages with respect to cost, simplicity of operation, and ease of maintenance.

The basic principle is similar to that of the air-driven vacuum-centrifuge of the type which was first described by Bauer and Pickels (1936), and which was based upon developmental work by Beams and his associates (1935) based on the principle of Henriot and Huguenard (1925). A conical rotor, R (fig. 1), carrying the material under investigation, is suspended and spun inside an evacuated chamber, C, by means of a flexible shaft, S, which passes to the outside through a sleeve-bearing, B, sealed by oil. This bearing is flexibly mounted on two Neoprene rings, D.

The essential modification which has been introduced in the present apparatus is to support and spin the rotor by a unit driven by an electric motor. The driving mechanism is a "lathe-grinder,"[3] which consists of a spindle mounted in ball bearings and belt-driven by a series-universal motor, M, which develops $\frac{1}{2}$ horsepower at its normal operating speed of 12,000 r.p.m. The maximal spindle speed is 44,000 r.p.m., obtained by the use of a suitable combination of pulleys. We have employed a rheostat for intermediate control. The whole lathe-grinder is compact and is mounted vertically atop the vacuum chamber. The lower end

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[3] We have used a No. 5 lathe-grinder, manufactured by the Dumore Company, Racine, Wisconsin. The lubricating system was slightly modified to permit vertical operation.

The authors are prepared to supply, upon request, the details of the construction of the ultracentrifuge.
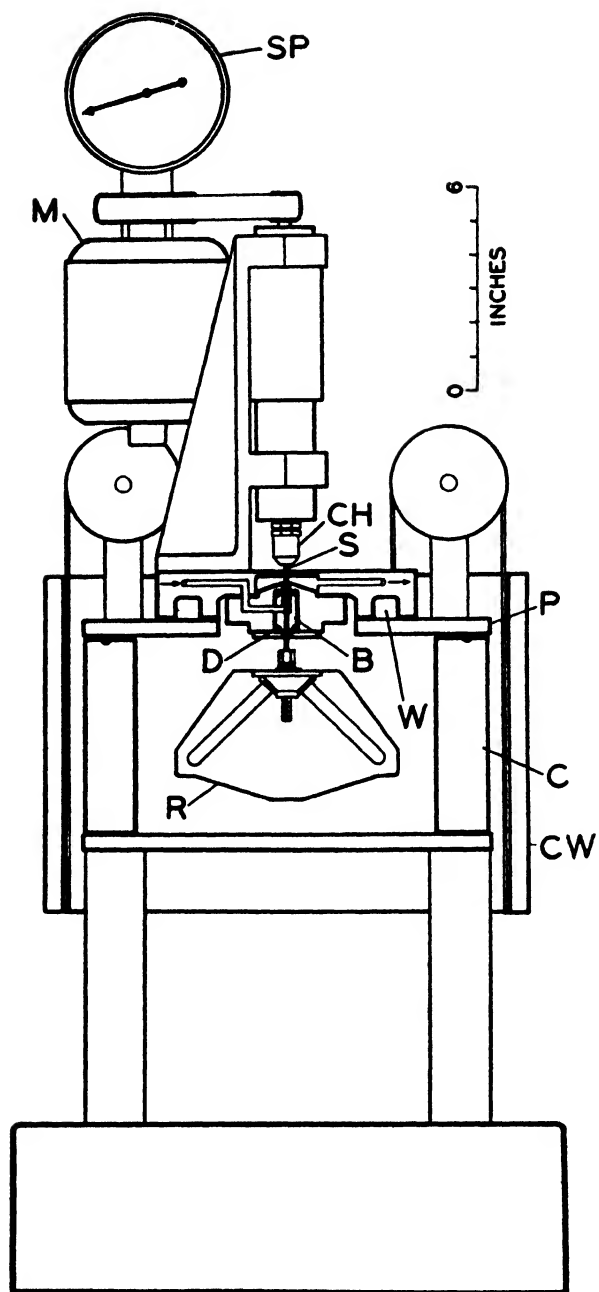
FIG. 1. SCHEMATIC DIAGRAM OF THE MOTOR-DRIVEN ULTRACENTRIFUGE

of the spindle is equipped with a chuck, *CH*, into which is fastened the flexible shaft bearing the rotor.

It has been necessary to provide means for cooling the vacuum-tight bearing. A small gear-pump, operated by the motor which also drives the vacuum-pump, forces lubricating oil through a copper coil cooled by running water, and thence causes it to circulate around the bearing, as indicated by the arrows. The top plate and bearing are also cooled by water circulating in the channel, *W*. Leakage of oil through the bearing has been too slight to necessitate a collecting device within the vacuum-chamber.

The rotor was made from Dowmetal, because of its low density, the ease with which it can be machined, and its high resistance to fatigue. Although Dowmetal is very susceptible to corrosion, it was felt that its other properties warranted its use, despite the necessity for occasional polishing.

The mounting of the centrifuge is simple. The square plate, *P*, which forms the top of the vacuum-chamber, *C*, and which carries the driving mechanism, is mounted at the corners on posts, two of which are indicated in figure 1. The posts are embedded in a base of concrete. The chamber proper is free to move vertically and is suspended by small cables fastened to the bottom plate, the corners of which are cut away in order to clear the supporting posts. The suspending cables pass over pulleys, as shown, and are attached at their other ends to the bottom edge of an outer shield of boiler-plate, *CW*, which serves also as a counterweight. Thus, in order to attach or remove the rotor, the outer shield is raised, whereupon the vacuum-chamber is lowered, the proportions being such that a sufficient gap results to permit ready access to the rotor.

The speed of the centrifuge is measured by means of an automobile speedometer, *SP*, permanently connected to the motor by gears in such a ratio that each division of the scale represents 1,000 revolutions of the rotor per minute. Tests made with a stroboscope have shown that this method is sufficiently accurate for quantity-centrifugation, and that the slippage in the belt-and-pulley driving mechanism is insignificant.

The results of numerous runs, made under various conditions

during the past year, justify the following statements concerning the operation of the centrifuge. At speeds up to 33,000 r.p.m. for periods of as long as 3 hours, the temperature of the rotor does not rise more than 2 degrees. From 5 to 10 minutes are required to attain a speed of 33,000 r.p.m., after which the speed remains constant within ± 2 per cent. The vacuum necessary for operation is likewise attained within 10 minutes. The centrifuge comes to a stop from 33,000 r.p.m. within 5 minutes after the power is shut off. Service requirements have been minimal, consisting of lubrication, occasional replacement of the belt, and replacement of the flexible shaft after from 6 to 8 hours of use. Interchangeability of the shafts, without individual machining, was achieved through the use of $\frac{1}{8}$-inch drill-rod.

When the centrifuge has been operated at its maximal speed, 44,000 r.p.m., for periods of from 3 to 4 hours, the temperature of the rotor has usually risen 8 or 9°C. This rise appears to be attributable to insufficient evacuation, for the pressure within the chamber has varied from 10 to 100 microns in most of the runs, whereas it should be not more than 1 micron, according to Beams, Linke and Sommer (1938). Although the rise in temperature is no greater than that which develops in ordinary centrifuges during prolonged runs, it constitutes an undesirable feature which has still to be eliminated.

The following two protocols of typical experiments demonstrate that sedimentation of biological materials by the present centrifuge has occurred according to expectation. In all cases, celluloid tubes (3 x $\frac{1}{2}$ inch) have been used in a rotor of $6\frac{7}{16}$ inches outside diameter, drilled to carry 6 tubes at an angle of 45°.[4] The capacity was 7 ml. per tube.

Experiment 1. Sedimentation of influenza virus. A 10 per cent suspension of influenzal mouse lungs, infected with strain WS, was prepared in broth, centrifuged, and the supernate filtered through Whatman No. 50 paper. A portion of the filtrate was spun at 32,000 r.p.m. for $1\frac{1}{2}$ hours. The sediment was suspended in the original volume of broth, coarse aggregates were removed by horizontal centrifugation, and the suspension was again centri-

[4] This rotor has recently been superseded by one of similar dimensions, but designed to carry 16 of the tubes at an angle of 22.5° from the vertical axis.

fuged as before. The final sediment was homogenized in the original volume of broth. The results of the tests of infectivity for mice, recorded in table 1, show that the virus was very largely sedimented from the original filtrate and was recovered in undiminished potency in the final sediment. Similar results have been obtained when sedimentation was carried out at from 15,000 to 18,000 r.p.m. for $1\frac{1}{2}$ hours.

TABLE 1

Sedimentation of influenza virus.   Infectivity of fractions for mice

DILUTION	STARTING MATERIAL			SUPERNATE			FINAL SEDIMENT		
10^1	D5	D5	3+	2+	2+	1+	D5	D5	D5
10^2	D5	D5	2+	2+	1+	±	D5	D6	3+
10^3	3+	3+	2+	1+	0	0	D6	3+	2+
10^4	2+	2+	1+				3+	2+	0
10^5	2+	1+	1+				1+	1+	0

Each dilution was tested in three mice.   D5 = death in 5 days.   1+, 2+, 3+ represent increasing degrees of consolidation of lungs (4+ would represent complete consolidation).

TABLE 2

Sedimentation of edestin and of concentrated globulin from diphtheric antitoxin. Nitrogen content of the various fractions

FRACTION	EDESTIN		DIPHTHERIC GLOBULIN	
	mgm. N	*per cent*	*mgm. N*	*per cent*
Top third of supernate..	0.00	0	2.77	9.0
Middle third of supernate	0.81	8	5.38	17.5
Bottom third of supernate	3.14	31	15.19	49.5
Sediment	5.94	59	7.52	24.5
Total recovered	9.89	98	30.86	100.5

Experiment 2.   Sedimentation of proteins. Two proteic solutions were tested, first a 1 per cent solution of edestin (molecular weight = 309,000 (Svedberg, *et al.*, 1939)) in 10 per cent sodium chloride and second, a 1:6 dilution, in 0.85 per cent sodium chloride, of a commercial, concentrated equine globulin prepared from diphtheric antitoxin. Portions of these solutions were spun at 44,000 r.p.m. for 4 hours, during which time the temperature of the rotor rose 9° above that of the room. The supernates were carefully withdrawn by thirds and the sediments were

dissolved in their respective solvents. Aliquots of all fractions were analyzed for nitrogen by a micro-Kjeldahl procedure. The results, recorded in table 2, show that marked sedimentation of the proteins occurred, especially in the case of edestin, where 59 per cent separated in the sediment, and 31 per cent in the bottom third of the supernate.

The present centrifuge, therefore, has been shown to be capable of sedimenting, within a reasonable time, substances of molecular weight as low as 309,000.

The authors wish to acknowledge their indebtedness to the late Charles H. Shaw, Esq., for invaluable suggestions, especially in regard to the present use of a lathe-grinder.

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STUDIES ON SOME STRAINS OF BUTYRIC-ACID-PRODUCING PLECTRIDIA ISOLATED FROM HEMP, JUTE AND FLAX

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INTRODUCTION

During isolations of the so-called butyric and butyl anaerobes from hemp, jute and flax obtained from various countries, plectridial forms of a butyric-acid-producing organism were obtained from all three types of vegetable fibers. These plectridia are able to ferment certain carbohydrates with the production of carbon-dioxide and hydrogen along with butyric, acetic and lactic acids and slight amounts of acetone, isopropyl, butyl and ethyl alcohols. Certain strains of the plectridia were easily distinguishable from the other butyric acid organisms present by their characteristic colonial form on nutrient agar slopes. As with butyric organisms in general no specialized nutrient media appear to be required for growth. On an ordinarily suitable medium the butyric organisms present on the fibers develop to such an extent that they tend to suppress other organisms and can be isolated with comparative ease.

To isolate these bacteria, pieces of well-washed fiber material were put into tubes containing maize mash or milk, and anaerobically sealed according to Dorner and Ritter's method (Dorner and Ritter, 1933), and cultured at 37°C. Within 24 hours or so active gassing took place. Those tubes were selected which showed the most vigorous evolution of gas.

For the isolation and purification of these organisms, smear cultures were made on yeast-glucose-agar slopes. The tubes were then anaerobically sealed and incubated at 37°C.

Generally within 24 to 48 hours various types of well developed colonies were formed. These colonies could be easily picked at and further cultured in the same manner.

The plectridia generally develop large, rounded, dirty-white, glutinous, compact colonies. A slimy substance which has been produced binds the cells together so closely as to form a stout pellicle, which, under conditions of very active growth, is thrown into folds giving an irregular often crested star-shaped form (see fig. 1). The entire colony whether smooth or crested, comes

Fig. 1. Typical Colony of C pectinovorum (Isolated from Indian Hemp) 48 hours growth on yeast-glucose-agar under anaerobic conditions at 37°C.

away from the solid medium when picked at with the inoculating needle. These features aid in distinguishing these plectridia from the clostridial form of butyrics. The latter are generally creamy-colored, smooth and often butyrous and may in certain cases form short slimy threads when picked at; but the whole colony is not removed, almost intact, as in the former case.

Two strains of plectridia isolated from Palestine jute and Indian hemp exhibited the above-described form of colony. One strain of plectridia isolated from Indian flax did not, however, produce this type of colony form. Instead, small, rounded, semi-trans-

parent rather punctiform-like colonies were produced, which have remained a constant feature of this strain for a period of more than four years and consequently can be considered a distinguishing feature for this particular type of plectridium.

A study has been made of these strains and their systematic position considered.

For the sake of brevity and a clearer comparison of the three strains of plectridia isolated from Palestine jute, Indian hemp and Indian flax, their diagnoses are given under one descriptive chart. The strains are indexed as PJ, IH, and IF, respectively.

Descriptive chart

Name: Plectridium species (*Clostridium pectinovorum*).
Source: Palestine jute, PJ; Indian hemp, IH; Indian flax, IF.

Morphology

Vegetative cells: Form: short and long, somewhat curved, rods. Arrangement: single or short chains. Ends: rounded.

Sporangia: Present. Form: spatulate to capitate.

Endospores: Present. Location: terminal. Form: oval. Wall: thick, adherent in PJ, IH, but thick and non-adherent in IF.

Mobility: Vegetative rods motile.

Flagella: Present, peritrichous. Stain: Cesares-Gil.

Capsule: Present. Method: Gin's Indian ink and Carbol-fuchsin.

Staining reactions: Gram-positive in young cultures. Lugol's iodine: young vegetative cells yellow, granulose in plectridia stains blue-violet. Young plectridia stain more readily than older plectridia. Congo red: no red staining of granules observed.

Cultural characteristics

Aerobic agar streak: No growth on yeast-glucose-agar.

Anaerobic agar streak: Medium: (1) Yeast-agar slopes. Growth slight but clearly visible within 24 hours at 37°C. Colonies: minute, rounded, transparent.

(2) Yeast-glucose-agar slopes. Growth: visible within 24 hours at 37°C. Colonies: PJ and IH, large, rounded or irregular raised, smooth or crested often star-shaped, somewhat hard, compact, glutinous, wax-like, colonies from several millimeters, up to about 5 mm. diameter within 48 hours. Whole colony removed with inoculating needle. IF,

colonies are small, circular, slightly raised, translucent, smooth, entire margin about 1 mm. diameter.

Maize mash: Fermentation: rapid. Diastatic action: partial, rapid abundant gas production. So-called "head" collapses after some time. Odor: generally butyric.

Potato mash: Similar to maize.

Milk: Coagulation: rapid. Coagulum: broken up through active gassing; well peptonized, acid reaction.

TABLE 1

Measurements of vegetative cells, etc., on yeast-glucose-agar medium at 37°C.

STRAIN	LIMITS OF SIZE		AVERAGE SIZE	
	Length	Width	Length	Width
Vegetative cells				
	microns	*microns*	*microns*	*microns*
PJ	1.7–7.0	0.4–0.6	4.2	0.5
IH	2.2–6.3	0.5–0.7	3.8	0.6
IF	1.7–9.1	0.4–0.7	3.7	0.6
Sporangia				
PJ	5.8–10.0		6.9	
IH	6.0–10.0		7.7	
IF	5.6–14.2		7.8	
Width of tail varies from about 0.7 microns to spore width				
Endospores				
PJ	1.4–2.7	1.0–1.8	2.1	1.43
IH	1.7–2.8	1.1–1.6	2.3	1.4
IF	1.2–2.3	1.0–1.6	1.8	1.2

Nitrate reduction: Medium: glucose-nitrate-peptone broth. Test: dimethyl-α-naphthylamine sulphanilic acid. Nitrite: negative.

Temperature range of fermentation: 20–45°C. Optimum: 37°C.

Gelatin liquefaction: Medium: glucose-gelatin. Liquefaction: positive within several days.

Production of H_2S: Medium: (1) Yeast-glucose-agar with lead acetate paper. H_2S; slight traces shown by PJ, IH and IF. (2) Yeast-glucose-lead acetate agar; H_2S not detected.

Catalase production: Cultures used: good growth on yeast-glucose-agar slopes under anaerobic conditions. Three per cent H_2O_2 not decomposed, showing absence of catalase.

Medium and apparatus. Fifty-milliliter flasks with 35 ml. of nutrient medium containing 0.05 per cent specially prepared yeast autolysate, 0.0375 per cent asparagine, 0.2 gram asbestos, plus 1.5 per cent (unless otherwise stated) carbohydrate or related substance. Flask connected with a graduated gas burette according to the method of Dr. Rosenfeld of our Institute (Weizmann and Rosenfeld, 1937).

Incubation temperature. 37°C.

Inoculant. Colonies of the organisms were at first used for inoculation purposes, so as not to introduce any extraneous carbohydrate material along with the inoculant, but subsequent growth was not always reliable; therefore 0.5 ml. of 5 per cent maize mash culture used instead.

Method of observation. Gas production, titratable acidity and where possible fermented carbohydrate. The results are given in table 2.

Carbon metabolism

It is evident from table 2, that the majority of carbohydrates, such as sucrose, glucose, lactose, maltose, galactose, arabinose, starch, etc., are easily attacked by these organisms. Esculin and mannitol are quite unsuitable as a carbon source, whereas glycerol, inulin, pectin, cellulose and calcium lactate are only slightly attacked, if at all. It should be mentioned that in a number of experiments rhamnose was hardly attacked, although in the instances recorded rhamnose was attacked. In considering these results one must not overlook the fact that slight metabolism was shown to occur when these organisms were put on to the basal medium containing yeast autolysate and asparagine, which are especially used to facilitate favorable conditions necessary for growth and fermentation. This was further confirmed by a slight but noticeable growth of these strains on agar slants containing only 0.5 per cent Difco's yeast extract.

Analyses of the products of fermentation of glucose

A quantitative comparison was made of the products of fermentation of glucose by these plectridia. In the first series of

experiments (table 3), medium I used consisted of 1.0 gram specially prepared yeast autolysate, 0.375 gram asparagine, 20 gram glucose made up to 1 liter with tap water plus 2 gram asbestos. The inoculant for each liter experiment was an active

TABLE 2

Fermentation of various carbohydrates, alcohols and glucosides after 7 days at 37°C.

Medium: 35 ml. of yeast asparagine medium plus about 1.5 per cent carbohydrate or similar substance.

	N/10 TITRATABLE ACIDITY OF 10 ML. FERMENTED MEDIUM			GAS PRODUCTION		
	PJ	IH	IF	PJ	IH	IF
	ml.	ml.	ml.	ml.	ml.	ml.
Glucose........................	1.45	2.35	1.08	56	64	45
Lactose........................	1.50	1.65	0.90	28	50	20
Sucrose......	1.70	1.90	0.90	46	53	24
Maltose...	1.25	1.50	0.60	30	43	15
Galactose	1.40	1.55	0.65	41	51	24
Raffinose.......	1.30	1.85	0.55	24	48	20
Levulose.....	1.50	1.90	0.85	52	58	20
Arabinose	1.50	1.85	0.70	34	54	20
Xylose........	0.50	1.55	0.90	10	42	20
Rhamnose.	0.40	1.60	0.50	8	29	14
Mannose........	1.85	1.75	0.80	47	51	20
Starch...	1.45	1.85	1.08	42	50	35
Glycerol.....	0.60	0.30	0.25	10	14	10
Pectin (0.5 per cent).	0.35	0.36	0.52	10	12	17
Dextrin......	1.25	1.90	1.20	28	46	20
Inulin....................... ...	0.40	0.35	0.35	8	6	6
Glycogen........	2.0	2.50	1.70	43	56	30
Salicin........	1.15	1.80	0.10	29	45	9
Mannitol	0	0	?	0	0	?
Sorbitol.....	0.75	0.85	0.60	12	15	15
Esculin (0.5 per cent)	0	0	0	0	0	0
Erythrite (0.5 per cent).....	1.05	1.30	1.05	18	21	14
Cellulose..	0.60	0.60	0.50	12	15	12
Ca. lactate.	0.20	0.50	0.35	3	20	10
Basal medium + inoculant.	0.13	0.10	0.25	7	3	11

maize-tube culture 24 hours old prepared from single colonies of the organism grown anaerobically on yeast glucose agar slopes.

Subsequent experiments on glucose, series II and III (table 4) were carried out in the presence of chalk. In these experiments

TABLE 3

Products of fermentation in 1 liter yeast asparagine glucose medium after 6 to 7 days incubation at 37°C.

	PJ	IH	IF
Titratable acidity in ml. 0.1 N acid in 10 ml..	1.6	1.8	1.2
Glucose fermented, per cent......	16.3	20.5	9.0
	Products calculated in mMols per 100 mMols of glucose fermented		
Acetone...........	1.4	2.7	6.6
Isopropyl alcohol...........	5.2	6.6	9.8
Butyl alcohol	36.5	7.7	1.9
Ethyl alcohol...	5.2	55.9	92.1
Acetyl methyl carbinol..	0.0	0.0	0.0
Butyric acid.	36 3	62.6	34.9
Acetic acid.	22 5	19.0	85.6
Formic acid......	0.0	0.0	0.0
Lactic acid	10.4	0.8	21.3

TABLE 4

Fermentation products from 2 per cent glucose in the presence of 0.5 per cent Difco's yeast extract and CaCO₃

Series II: 1 liter medium
Series III: 350 ml. medium

EXPERIMENT SERIES	PJ		IH		IF	
	II	III	II	III	II	III
Glucose fermented per cent .	100	98.3	98.35	98.41	74.65	97.51
	Products calculated in mM per 100 mM of glucose fermented					
Butyric acid	23.9	43.0	39.5	50.5	9.0	16.0
Acetic acid.	43.2	55.8	26.4	50.5	22.6	28.0
Lactic acid.... ...	7.0	9.7	17.3	6.7	106.2	97.0
Formic acid	2.3	7.0	0.2	4.2	8.6	6.2
Acetone	13.8	2.0	4.3	0.7	3.2	0 8
Isopropyl alcohol..	2.1	4.5	1.6	2.9	5.9	0.3
Butyl alcohol	18.1	2.6	23.0	0.9	5.5	3.0
Ethyl alcohol	14.7	24.7	21.3	20.4	47.3	19.5
Acetyl methyl carbinol.... .	0.0	0.0	0.0	0.0	5.0	0.0

the asbestos was omitted and only 1 ml. of an active yeast-glucose culture of the organism was used per flask experiment.

All experiments were made at 37°C.

Analytical methods

Titratable acidity. The titratable acidity was determined by heating 10 ml. of fermented medium to boiling point and titrating with 0.1 N NaOH using phenolphthalein as indicator.

Unfermented sugar. Residual sugar was determined according to either Stiles, Peterson and Fred's (Stiles, Peterson and Fred, 1926) modification of the micro method of Shaffer and Hartmann, or Bertrand's method.

Solvents. In series I (table 3) 500 ml. neutralized fermented medium were distilled and 200 ml. distillate collected. In subsequent experiments, however, 200 ml. medium were distilled and 100 ml. distillate collected. Acetone was estimated according to Goodwin's modification (Goodwin, 1920) of Messinger's method; isopropylalcohol by the method recommended by Langlykke *et al.* (Langlykke, Peterson and McCoy, 1935) and ethyl and butyl alcohols by Johnson's (Johnson, 1932) method.

Volatile acids. In the first series of experiments the residue from the above distillation was acidified with sulphuric acid, steam distilled and 500 ml. distillate was collected. The volatile acids were determined according to Virtanen and Pulkki's (Virtanen and Pulkki, 1928) modification of the Duclaux method. In the experiments recorded in table 4, 50 ml. of the fermented medium were acidified with phosphoric acid and distilled to about 12 ml. Fifty milliliters of CO_2-free distilled water were added to the distilling flask and distillation continued. This was repeated until all volatile acids were distilled over. The acids were then determined according to Duclaux. The distillation constants were calculated from our distillation figures obtained with pure acids.

Formic acid. This was estimated directly on the fermented medium by the Fincke method (Official Methods, 1935).

Lactic acid. Friedemann and Graeser's (Friedemann and Graeser, 1933) method was used for the determination of lactic acid.

Acetyl methyl carbinol. This was determined on 200 ml. of the fermented medium according to Kluyver, Donker and Visser't Hooft's (1923) modification of Lemoigne's method. Table 3 shows that these organisms are fair fermenters of glucose, producing in general negligible yields of neutral volatile products as acetone, butyl and isopropyl alcohols; and fair amounts of ethyl alcohol and butyric, acetic and lactic acids.

An interesting observation from these experiments is that strain IF produced less volatile acids as butyric and acetic and markedly more lactic acid than strains PJ and IH. This was confirmed by further experiments (table 5). Strain IF in general also produced less butyl alcohol.

TABLE 5

Per cent lactic acid on fermented glucose in the presence of CaCO₃

EXPERIMENT SERIES	MEDIUM	PJ	IH	IF
IV	1 liter 2 per cent glucose yeast chalk.	3.5	7.8	24.8
V	900 ml. 1 per cent glucose caseinogen digest broth	9.5	13.8	51.6
VI	350 ml. 2 per cent glucose yeast chalk	8.1	4.3	32.9 28.6

Proteolytic activities on milk

The ability of these organisms to peptonize milk has already been referred to in the diagnosis chart of these organisms. The breakdown of the casein is just as rapid among these plectridia as by the closely related form *Clostridium acetobutylicum* which is also highly proteolytic on casein. Table 6 gives some quantitative information on the breakdown of milk casein by the strains PJ and IH. A comparison is also given with some available preliminary data on the production of amino N from milk proteins by *Clostridium acetobutylicum* (strain B.Y. of our collection).

Milk cultures of the organisms were inoculated into 700-ml. flasks containing 500 ml. milk. At certain intervals samples were carefully withdrawn and tested for titratable acidity and soluble and amino nitrogen.

TABLE 6
Proteolysis of casein in milk by strains PJ and IH

FERMENTATION PERIOD	STRAIN	TITRATABLE ACIDITY 0.1 N ACID IN 10 ML.	TOTAL SOLUBLE N		AMINO N	
			100 ml. fermented milk	Calculated as per cent on total N in milk (521.1 mgm. per 100 ml.)	100 ml. fermented milk	Calculated as per cent on total N in milk
		ml.	mgm.		mgm.	
0	PJ	2.0	64.0	12.3	20.1	3.9
	IH	2.0	60.4	11.4	20.0	3.8
24 hours	PJ	3.15	81.8	15.7	24.4	4.7
	IH	3.95	84.1	16.2		
48 hours	PJ	4.30	136.4	26.2	27.3	5.3
	IH	4.58	117.5	22.0	23.1	4.4
72 hours	PJ	5.13	185.4	35.6	36.0	6.9
	IH	4.68	139.2	26.7	37.6	7.2
6 days	PJ	5.65	219.0	42.0	53.0	10.2
	IH	5.0	172.8	33.2	42.8	8.2
10 days	PJ	6.53	243.5	46.7	69.2	13.3
	IH	6.28	212.2	40.7	77.4	14.9
15 days	PJ	9.67	271.9	52.2	77.1	14.8
16 days	IH	6.35	242.9	46.6		
23 days	IH	7.48	257.6	49.4		
36 days	IH	8.03	303.5	58.3		

Clostridium acetobutylicum (Weizmann's B.Y. strain) on milk produced the following amounts of amino nitrogen per 100 ml. milk (total N content = 521.1 mgm. per 100 ml.):

	Mgm.	Calculated as per cent on total N in milk
48 hours	20.1	3.9
72 hours	49.0	9.6
4 days	55.7	10.7
7 days	93.3	17.9
18 days	121.2	23.3

N-fixation

Our attempts to prove the N-fixation powers of our plectridia have so far given only negative results. Various media were tried including Winogradsky's N-free medium and McCoy and co-workers' (McCoy, Higley and Fred, 1928–29) synthetic medium containing 0.01 per cent peptone. Media containing somewhat greater amounts of nitrogen were used but no glucose fermentation or N-fixation could be detected.

These results are at variance with those obtained by several investigators working with similar if not identical plectridia. For instance McCoy and her associates (1928–29) give the N-fixation powers of certain plectridia similar to ours as 0.68 to 2.75 mgm. N fixed in 100 ml. of their synthetic medium. Sjolander and McCoy (1937) working with Ruschmann's *Plectridium pectinovorum* (Ruschmann and Bavendamm, 1925), also similar, if not identical with our strains, obtained a fixation of 0.33 to 0.80 mgm. nitrogen in 100 ml. medium.

Mention might be made of an early reference by Keutner (1904) to the N-fixation powers of his so-called *Paraplectrum* sp., found by him as a begleit-bacillus to *Clostridium pasteurianum* in raw cultures. Although uncertain regarding N-fixing by his plectridial organism, Keutner thought fixation possible. However, the begleit-bacillus is vaguely described so that a fair comparison with our plectridia cannot be made.

Retting and pectin fermentation

The fermentation of pectin is of special interest here as plectridia possessing remarkable retting abilities have been described by Winogradsky (Winogradsky and Friebes, 1895), Störmer (1904) Beijerinck and van Delden (1904) and Ruschmann and Bavendamm (1925).

The results of our experiments conducted on the fermentation of purified lemon pectin tend to show that our plectridia are able to bring about a slight amount of pectin fermentation (see table 2). From a number of trials, the IF strain appears to be definitely better than the other two.

Further tests were carried through on the fermentability of pectin under more or less natural conditions by these organisms. Test tubes with potato and carrot slopes partly immersed in water were autoclaved at 105°C. for one hour. Also, hemp strands about 8 cm. long were put into test tubes containing 9 ml. of water and 1 ml. of our yeast-asparagine-glucose medium to encourage the better development of the organisms. The hemp was steam-sterilized for ¼ hour on three successive days. Within a week, strain IF began to cause a slightly perceptible breakdown of the parenchymatous potato tissue. This breakdown progressed very slowly indeed. By the 20th day, although far from complete, the organism had produced a considerable flocculence of the potato tissue as a result of the separation of a certain amount of the cells through the breakdown of the middle lamella holding the cells together. The IH strain yielded only a slight breakdown, and strain PJ was negative in its action on the pectinous middle lamella of the cell walls. During the same period the carrot tissue remained intact, and no visible signs of retting of the hemp could be detected.

We do not wish to enter into a discussion here on the use of these organisms as retting agents. For the present we merely record that slight pectin fermentation has been observed by us with our strain IF and hardly any, if any, by the other two strains. McCoy and associates (1935, 1937) too, obtained hardly any fermentation of pectin by their strains isolated from soil and with Ruschmann's strain isolated from hemp. They point out (McCoy and Peterson, 1928) that much confusion in the literature on pectin fermentation is due to the use of impure commercial pectins. On the other hand Störmer (1904) and Ruschmann and Bavendamm (1925) regard their plectridia as important retting agents.

Nomenclature and taxonomy

In attempting to identify and name the plectridia we have isolated from hemp, jute and flax, it is obvious from our data that they are closely allied to the two better-known groups of butyric-acid-producing organisms, namely, the *Clostridium aceto-*

butylicum Weizmann group better known as the butyl organisms, and the *Clostridium butyricum* Prazmowski group. The plectridia group, however, is markedly different from the butyricum and acetobutylicum groups on morphological and physiological lines. Their fluted often star-shaped, glutinous, semi-translucent colonies on yeast-glucose-agar slopes under anaerobic conditions single them out from the other butyrics. Peculiar too are their spatulate and capitate sporangia resembling tennis rackets or
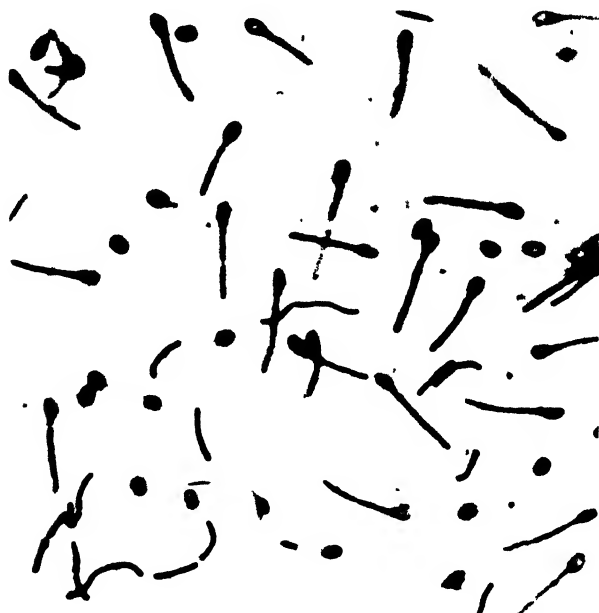
Fig. 2 C. PECTINOVORUM (STRAIN PJ) ON Y.D AGAR
48 hours growth at 37° (× 1440)

drum-sticks. Similar to *C. acetobutylicum* these organisms are strongly proteolytic, and liquefy gelatin, a property not possessed by the butyricum group. But unlike *C. acetobutylicum* they produce negligible quantities of acetone and butyl alcohol from maize mashes, in this respect resembling the butyricum-group. In short from the systematic standpoint they form a natural group between *C. acetobutylicum* and *C. butyricum*.

Reviewing the literature, plectridia which appear to resemble

ours have been mainly described as retting organisms. The first detailed account of a retting plectridium with which ours may be compared both morphologically and in many of its physiological features, is the Friebes organism described by Winogradsky in 1895. This organism is in all probability, despite the smaller spore measurements recorded, the same as that more fully described by Störmer under the name of *Plectridium pectinovorum*. *Plectridium pectinovorum* and our plectridia

FIG. 3. C PECTINOVORUM VAR. PARVUM (STRAIN 1F) ON MAIZE
48 hours growth at 37° (× 1440)

readily liquefy gelatin; because of this Beijerinck and van Delden's (1904) retting plectridium, *Granulobacter pectinovorum*, a non liquefier of gelatin, does not fall within this group.

Our plectridia and the retting plectridia also differ physiologically from Omelianski's active cellulose-fermenting plectridium (1902) which produces appreciable yields of butyric and acetic acids from cellulose, and is said to be unable to attack even the most readily fermentable carbohydrates.

There are several outstanding differences between our organ-

isms and *Paraplectrum foetidum* Weigmann (1898) with which there might perhaps be a certain systematic relationship. Weigmann observed that his organism has a lichenous colonial form and exerts a strong proteolytic action on milk casein, and as its name suggests, produces a strong smell like that of ripe Limburger cheese. The organism is stated to be gram-negative. Our organisms on the contrary are gram-positive, have a raised crested colonial form (strain IF however produces small semitranslucent rounded colonies) and produce butyric acid and negligible or slight amounts of acetone and butyl alcohol from carbohydrate media. With regard to the terminology *Paraplectrum*, Weigmann follows Fischer's nomenclature for these tadpole-like or drumstick-shaped sporangia, reserving the name *Clostridum* for those organisms which have spindle or ellipticalformed sporangia. It is, however, difficult to keep strictly to such terminology because of the known variations in sporangial forms even within one strain. For instance the form pictured by Weigmann as *Paraplectrum foetidum* bears close resemblance to some of the forms found within a single strain of *Clostridium butyricum*.

Our organisms approach very closely Störmer's description of his *Plectridium pectinovorum* and Ruschmann & Bavendamm's *Clostridium amylobacter* (*liquefaciens*) (Syn. *P. pectinovorum*). We would without hesitation regard them as identical, were it not for the fact that these authors consider their organisms as active retting agents, hence the specific nomenclature, whereas from our experience to date we could only consider one of our three strains, namely IF, as a slight to fair pectin fermenter. In respect of two of our strains we agree more, although not absolutely with, Sjolander & McCoy who working with Ruschmann's *Plectridium pectinovorum* (*liquefaciens*) found that these organisms were actually unable to utilize pectin in artificial media. Under the natural conditions of retting these organisms may perhaps actually play an important rôle in the process.

McCoy and co-workers have studied in great detail a number of plectridia isolated from soil. On morphological and physiological grounds, two of our strains of plectridia are practically

identical with those of the American workers, and since Sjolander and McCoy found Ruschmann's *Plectridium* unable to ferment pectin, thus bringing it into line with their own strains and with ours, one should perhaps consider McCoy's and our plectridia as belonging to the group *Plectridium pectinovorum* Störmer.

We would however suggest a change in nomenclature which would bring these organisms under the same generic name of *Clostridium* along with *Clostridium acetobutylicum* Weizmann and *Clostridium butyricum* Prazmowski, with which they are so closely related as to appear to be a transition group from one species to the other. This generic change would conform with the decision of the Society of American Bacteriologists (Winslow et al., 1917, 1920) and also Bergey (1934) that *Plectridium* is not sufficiently distinct to warrant generic differentiation.

Störmer's specific name *"pectinovorum"* could still be retained since the organism has gained some repute under this specific name, even though we may question its power to ferment pure pectin. Strains PJ and IH may be considered as strains of the type species *C. pectinovorum* Störmer; in this particular instance IH is more active than PJ. Strain IF, however, can be readily distinguished from the others by its colonial form, by its marked ability to produce greater yields of lactic acid from glucose than our strains PJ and IH, and also Störmer's organisms, and perhaps by its fair fermentability of pectin. It can, therefore, be considered as a distinct variety within the species. We propose tentatively the varietal name of *parvum* because of its fairly small transparent colonies.

SUMMARY

1. A detailed morphological and cultural diagnosis is given of certain strains of butyric-acid-producing plectridia isolated from Palestine jute, Indian hemp and flax.

2. These organisms form a natural group intermediate between the group *Clostridium butyricum* Prazmowski the true butyric clostridia, and the group of butyl organisms, *Clostridium acetobutylicum* Weizmann.

3. The fermentative abilities of these organisms on various

carbohydrates, alcohols, etc., were studied, and analytical estimations made of the fermentation products of glucose contained in various nutrient media.

4. These organisms ferment glucose, producing in general negligible yields of neutral volatile products as acetone, butyl and isopropyl alcohol, and fair amounts of ethyl alcohol, butyric, acetic and lactic acids. Strain IF, however, produces considerably more lactic acid then strains PJ and IH.

5. These plectridia are highly proteolytic on milk casein, causing a rapid solubilization of the casein with large amounts of amino nitrogen.

6. The organisms do not fix atmospheric nitrogen under conditions tested.

7. They may be considered slight to fair fermenters of pectin, but cannot be regarded as active pectin fermenters and consequently good retting agents, as were the retting plectridia described by Störmer and Ruschmann.

8. Their nomenclature and taxonomy are discussed, and reasons given for classifying them as belonging to the group *Clostridium pectinovorum* Störmer (Syn. *Plectridium pectinovorum* Störmer). One strain, readily distinguishable from the others by its greater production of lactic acid and its small colonial form on nutrient agar, is given the varietal name of *"parvum."*

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THE ENDOGENOUS RESPIRATION OF BACILLUS CEREUS

III. The Changes in the Rate of Respiration Caused by Sodium Chloride, in Relation to Hydrogen-Ion Concentration

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I. INTRODUCTION

It has been shown that most salts stimulate respiration in low concentrations, and inhibit it in higher concentrations (Ingram, 1939b). In the latter case the rate of absorption of oxygen usually varies with salt concentration according to the equation

$$\log r = P - Q.c \dots\dots\dots\dots\dots\dots\dots(1)$$

(where r is the rate of respiration expressed as a percentage of that in the absence of salt, c is the concentration of salt, and P and Q are constant for any one salt). Anomalous data obtained in certain cases were tentatively attributed to changes of pH during an experiment.

The present paper is a more detailed study of the pH relations of the system, using a single salt, sodium chloride; this salt is typical of many, which act in a similar way (Ingram, 1939b). The paper also gives an account of a similar investigation carried out using *Escherichia coli*. This organism is gram-negative, and has an endogenous respiration which varies greatly with hydrogen-ion concentration. It was hoped that the alterations produced in this respiration by salts might themselves change

683

considerably with hydrogen-ion concentration, and so be more easily perceptible than in the case of *Bacillus cereus*.

II. EXPERIMENTAL METHODS

The same strain of *B. cereus* was used in the same way as in previous investigations. Details of the procedure are given elsewhere (Ingram, 1939a and b). A similar procedure was followed with *E. coli*, which was grown on tryptone agar for 24 hours at 37°C.

The strain of *E. coli* used was no. 692 of the Type cultures of the Lister Institute. It was found to possess a small endogenous respiration which decayed quickly. Such behaviour is generally characteristic of gram-negative bacteria (Sevag, 1933).[1] The fall in the rate of respiration was especially marked during the first hour following the preparation of the suspensions. Thus, the rates of respiration were measured over a later period, $2\frac{1}{2}$ to $4\frac{1}{2}$ hours after the preparation of the suspensions, and all the rates given below represent averages over this interval.

The control and measurement of pH in the present experiments require special mention. The buffer most generally used was a mixture of KH_2PO_4 and K_2HPO_4 in 0.2 M concentration, but to obtain extreme reactions 0.1 M potassium phthalate and 0.1 M sodium borate were employed. The cells were washed and suspended in one of these solutions, and the suspension was then mixed with an equal volume of the appropriate concentration of sodium chloride solution. "Analar" reagents were used, and water distilled from Pyrex glass. The pH of each suspension, mixed with the salt solution, was measured at the end of the respiration measurements, that is, after about five hours. The determinations were made with a glass electrode, against standard buffers, and may have a uniform error of ±0.02 pH-units, being accurate among themselves to ±0.02 pH-units. Experiments were carried out in duplicate, and each datum represents the average of a pair of determinations.

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[1] Some feebly gram-positive bacteria behave in the same way, e.g., *Streptococcus lactis* (Callow, 1924) and butyric acid bacilli (Sevag, 1933). In the latter case, loss of endogenous respiration is accompanied by loss of the gram-positive character, owing to oxidative autolysis.

III. EXPERIMENTAL DATA

The changes of pH which occurred in the suspension

During the manipulation of suspensions in the way described above, there were factors in operation which tended to make the pH of the suspension differ from that of the buffer solution from which it was prepared.

In the first place, the cells themselves exerted a buffering action about a pH approximately that which they impart to an aqueous

TABLE 1

The changes of pH which occur when a suspension of bacterial cells is added to buffer solutions at 25°C.

BUFFER SOLUTION		B. CEREUS = 50 MGM. DRY WEIGHT PER ML.				E. COLI = 30 MGM. DRY WEIGHT PER ML.			
		pH of mixture after		Change of pH		pH of mixture after		Change of pH	
Buffer salt	pH of buffer	2 hours	5 hours	0-2 hours	2-5 hours	1 hour	5 hours	0-1 hour	1-5 hours
0.05 M phthalate	3.97*	4.30	4.42	+0.33	+0.12	4.30	4.32	+0.33	+0.02
0.1 M phosphate	5.50	5.54	5.56	+0.04	+0.02	5.56	5.63	+0.06	+0.07
0.1 M phosphate.	6.00	6.02	6.08	+0.02	+0.06	6.10	6.10	+0.10	0.00
0.1 M phosphate	6.50	6.50	6.44	0.00	−0.06	6.50	6.52	0.00	+0.02
0.1 M phosphate	7.00	6.96	6.86	−0.04	−0.10	6.96	6.95	−0.04	−0.01
0.1 M phosphate	7.50	7.36	7.26	−0.14	−0.10	7.44	7.44	−0.06	0.00
0.1 M phosphate	8.90*	8.62	8.55	−0.28	−0.07	8.70	8.68	−0.20	−0.02
0.05 M borate .	9.20*	8.96	8.94	−0.24	−0.02	9.04	8.94	−0.16	−0.10
		pH of equivalent aqueous suspension = 6.15				pH of equivalent aqueous suspension = 6.35			

* Solutions of low buffering power.

suspension; and this resulted in a change of pH, from that of the buffer towards that of an aqueous suspension. This is shown in table 1. The reaction of each buffer solution was moved towards a pH of about 6.2 by cells of *B. cereus*, and towards a pH of about 6.5 by cells of *E. coli*. However, the change was small after the first two hours, except in the most alkaline solutions. Table 1 shows also that the shift of pH was greater, the more the reaction of the buffer differed from that of an aqueous bacterial suspension. This was probably due, in the main, to the low buffering power of

solutions with extreme reactions; although Shaughnessy and Winslow (1927) have shown that cells of *B. cereus* and *E. coli* give off carbon dioxide in response to an alkaline menstrum, and ammonia in response to an acid menstrum, which would enable the cells to bring about relatively large pH changes in solutions of reaction widely different from their own. The addition of sodium chloride introduced a further complication. There was an immediate increase in acidity due to the increased ionic strength of the solution. This change has been discussed by Green (1933); it was of serious magnitude with solutions containing more than 0.2 M sodium chloride. Moreover, the presence of the sodium chloride altered the buffering power of cells of *B. cereus* (Shaughnessy and Winslow, 1927), but the changes arising from this cause were small enough to be neglected.

The complexity of the system made it essential to rely on actual observation of the pH of each suspension. The observation was made at the end of the period of observed respiration. This gave a just estimate of the average pH of the suspension over the period during which the respiration was measured because, as has been shown above, the change in reaction following the mixture of the salt with the buffer, and the smaller changes brought about by the buffering action of the cells themselves, were practically completed before measurements of the respiration were begun.

The relation between pH and respiration

In unbuffered suspensions, low concentrations of sodium chloride increase respiration while high concentrations diminish it (Ingram, 1939b). Similar changes were found to occur in buffered suspensions, over the whole of the experimental range of hydrogen-ion concentration.

The concentrations at which the salt was stimulatory were always less than 0.2 M. This concentration of salt never produced a pH shift greater than 0.2 unit, as is shown by a representative series of determinations in table 2. Thus, this table gives the remainder of the data pertaining to the stimulation of respiration, without regard to the shift of pH brought about by the

salt.   Examination of the data for *E. coli* reveals the following two points:

1. The salt concentration which gave the maximum rate of respiration was least at the pH of optimum respiration.   Thus

TABLE 2

The rates of respiration by cells of E. coli and B. cereus suspended in dilute solutions of sodium chloride buffered at different pHs

E. coli:						
Molar concentration of sodium chloride.	0.000	0.0002	0.002	0.02	0.1	0.2
pH of suspension	5.62	5.73	5.67	5.62	5.60	5.42

Molar concentration of sodium chloride	Rate of respiration expressed as: (a) Q_{O_2} (b) a percentage of that in the absence of salt

	pH OF SUSPENSION IN BUFFER SOLUTION							
	5.62		6.55		7.45		8.97	
	Q_{O_2}	Per cent	Q_{O_2}	Per cent	Q_{O_2}	Per cent	Q_{O_2}	Per cent
E. coli								
0.000	1.65	100	3.88	100	7.79	100	4.10	100
0.0002	1.70	103			8.50	108	4.37	107
0.002	2.35	142	4.00	103	8.50	108	4.90	120
0.02	2.60	158	4.62	120	8.25	105	5.30	129
0.1	3.07	186	4.20	108	7.62	97	6.55	160
0.2	1.57	95	3.57	92	6.75	86	2.67	65

	5.55		6.05		6.45		7.30	
	Q_{O_2}	Per cent	Q_{O_2}	Per cent	Q_{O_2}	Per cent	Q_{O_2}	Per cent
B. cereus								
0.000	18.0	100	20.0	100	22.6	100	22.0	100
0.0002			21.0	105	25.0	111	22.4	102
0.002	19.8	110	23.4	117	25.0	111	23.6	107
0.02	22.4	124	22.4	112	23.2	107	26.4	120
0.2	17.0	94	21.0	105	23.8	104	20.6	94

Q_{O_2} = cu. mm. O_2 per mgm. dry weight per hour.

at pH 7.5 the rate of respiration was greatest with 0.005 M sodium chloride, whereas at pH 5.6 or 9 the maximum respiration occurred in a solution with 0.1 M sodium chloride.

2. The proportional increase in respiration brought about by

addition of sodium chloride was also least at the pH of optimum respiration. Here, the highest rate of respiration was 10 per cent greater than that in the absence of salt. At pH 5.6 the rate of respiration in buffer alone was about one-fifth of that at pH 7.5, but by the addition of 0.1 M sodium chloride the rate was increased some 80 per cent; conditions were similar at pH 9. The stimulation of respiration by salt was thus much greater at
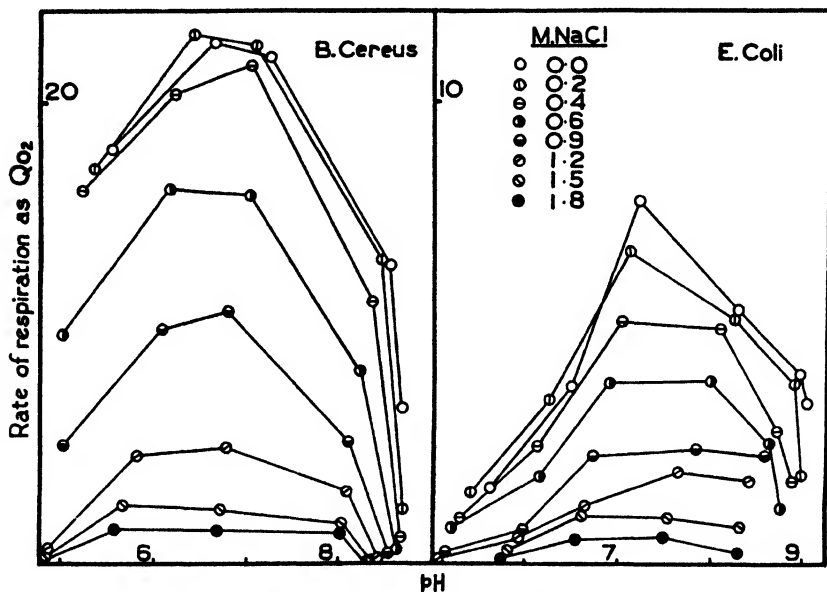
FIG. 1. THE RATE OF RESPIRATION BY CELLS OF B. CEREUS AND E. COLI, SUSPENDED IN BUFFERED SOLUTIONS OF SODIUM CHLORIDE

The increase in acidity, which results from the addition of salt to a buffered suspension, is clearly shown.

extreme pH than at the optimum; but it was inadequate to counteract the depressant effect of pH, so that the absolute values of the rate of respiration were never as great as that at the optimum pH of 7.5.

B. cereus behaves in the same way, but to a lesser degree. This is probably the result of the relative indifference of B. cereus to changes of hydrogen-ion concentration within the range investigated.

With concentrations of sodium chloride greater than 0.2 M, the shift of pH had to be taken into account, and its magnitude may be seen from figure 1, where the pH of each suspension is plotted against the rate of uptake of oxygen, for suspensions with various concentrations of sodium chloride. It is clear that the addition of sodium chloride did not change the pH of optimum respiration to any significant degree.

By the erection of ordinates at any pH on figure 1, one may calculate by interpolation the rates of respiration in solutions
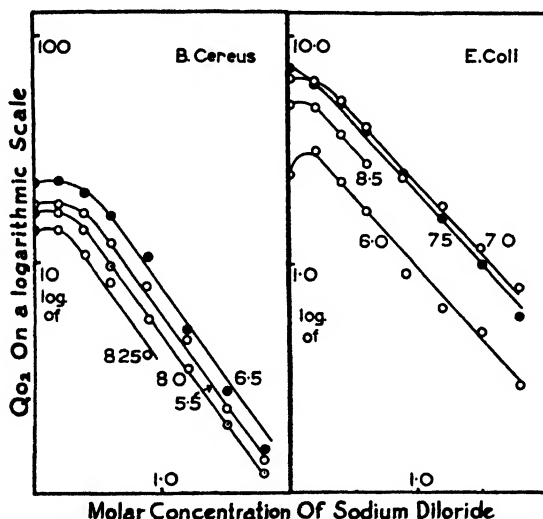
FIG. 2. THE RATES OF RESPIRATION BY CELLS OF B. CEREUS AND E. COLI, WHICH WOULD BE OBSERVED IN SALT SOLUTIONS BUFFERED AT CONSTANT pH, CALCULATED FROM FIG. 1

with differing salt concentrations but constant pH.   The results may then be used to test the validity of the relation

$$\log r = P - Q.c \dots\dots\dots\dots\dots\dots\dots (1)$$

r = rate of respiration
c = salt concentration
P and Q constant

which has only been shown to hold in unbuffered suspensions (Ingram, 1939b).   The results of this calculation are set out in figure 2.   The linear relation between $\log r$ and c is apparent,

and establishes equation (1) for both *B. cereus* and *E. coli*. This is true at any pH and moreover, the slope of the line relating log r to c, and hence the value of Q in this equation, is the same whatever the pH. (The logarithmic ordinate in figure 2 gives undue prominence to errors in the determination of the lower rates of respiration.) Q is about 0.75 for *B. cereus* and 0.60 for *E. coli*.

IV. DISCUSSION

One might be considerably misled, if the relation between respiration in buffers and concentration of added salt were expressed without regard to pH. For example, if suspensions of *B. cereus* in phosphate buffer of pH 5.5 mixed with different concentrations of sodium chloride are treated as a homogeneous series of constant pH, a curve such as that shown in figure 3 is obtained instead of the linear relation of figure 2. This happens because the rate of respiration is depressed not only by the salt, but also by the increased acidity which it causes; conversely, starting with a suspension on the alkaline side of the optimum pH, the rates of respiration in the more concentrated salt solutions appear high, because the shift of pH is in the direction of greater respiration in this case. Thus one might imagine that addition of salt caused a shift of the optimum pH in an alkaline direction, and that salt shows its greatest potency as an inhibitor of bacterial activity in acid solutions. However, with an organism such as *B. cereus* which has a broad respiration/pH optimum, an intermediate state is possible, in which the change of pH brought about by the addition of a salt is not great enough to alter the rate of respiration appreciably, and under such circumstances the relation expressed by equation (1) is not obscured if the pH is neglected (e.g., the curve for pH 7.5 on figure 3). The curve relating to suspensions in buffer of pH 5.5, shown on figure 3, is exactly similar to that presented by Nicolai (1926) in his study of the respiration of *E. coli* in phosphate buffers, and to data obtained with buffered suspensions of *B. cereus* by the present writer (Ingram, 1939b). It is clear that these earlier data have no significance because of the neglect of the latent changes of pH.

It was suggested (Ingram, 1939b) that the stimulating action of dilute salt solutions on the respiration of *B. cereus* was probably greater in buffered solutions of pH about 6.0 than in unbuffered solutions of pH about 6.15.   The data of table 2, in which a greater range of hydrogen-ion concentration is included, confirm this observation.   The increased stimulatory effect of dilute salt solutions with increasing acidity was easily perceptible at
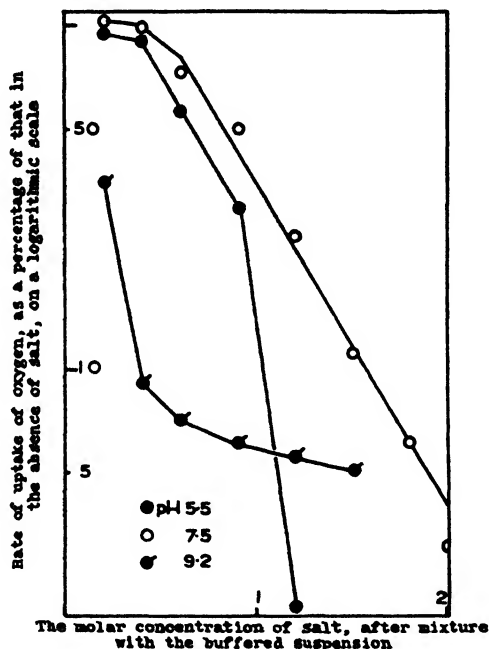
FIG. 3. THE RATES OF RESPIRATION ACTUALLY OBSERVED WHEN INCREASING CONCENTRATIONS OF SODIUM CHLORIDE ARE ADDED TO A SUSPENSION OF B. CEREUS, IN BUFFER SOLUTION OF INITAL pH 5.5, 7.5 OR 9.2

pH 5.6 with *E. coli*, as the divergence from the pH optimum was considerable. In addition, a similar increase was apparent at pH 9.   Thus the stimulation of respiration by sodium chloride, and the concentration of the salt necessary to produce it, were both least at the pH of optimum respiration and both increased on either side of this pH.   This is borne out by the behaviour of the curves on figure 2, at low concentrations of sodium chloride.   Moreover, in this figure the whole of each

curve falls lower the greater the divergence from the optimum pH, whether this divergence is on the acid or the alkaline side. In fact the effects of salt, both stimulatory and inhibitory, were symmetrical about the pH of optimum respiration.

The value of Q given above for *B. cereus* (0.75) is appreciably greater than that reported earlier (0.68—Ingram, 1938b). This organism was originally isolated from a medium containing roughly 1.0 M sodium chloride, and has since been cultured in one with only 0.2 M sodium chloride. The decline in resistance

TABLE 3

A comparison of the effects of salt and pH on the growth and respiration of
E. coli. The rates of growth and of respiration are calculated as
percentages of those in the absence of salt

	pH		
	6.0	7.0	8.0
Growth:*			
Peptone.	100	100	100
Peptone + 0.2 M sodium chloride .	187	148	240
Peptone + 0.2 M sodium citrate .	67	70	47
Respiration:†			
0.1 M phosphate.	100	100	100
0.1 M phosphate + 0.1 M sodium chloride. .	140	102	110
0.1 M phosphate + 0.6 M sodium chloride .	68	58	50

  * Calculated from the data of Sherman and Holm, 1922. The pH of optimum growth was 6.8.
  † The pH of optimum respiration is about 7.5.

to salt during the nine months elapsing between the two measurements of Q suggests that the halotolerant character may be "adaptive."

It is interesting to compare, at various hydrogen-ion concentrations, the data expressing the action of sodium chloride on the respiration of *E. coli* with those of Sherman and Holm (1922) for the action of this salt on growth by *E. coli*.

Sherman and Holm arrayed their data against actual measurements (by indicators) of the pH of the medium; the data of table 3 were obtained by interpolation. Table 3 shows that the stimulation of growth by sodium chloride was least at the optimum

pH, just as is the case for respiration; so that low concentrations of salt have a similar broadening effect on the pH-optima of growth and respiration. The inhibition of growth caused by 0.2 M sodium citrate can be appreciated by the use of an analogy. It has been shown that, in its influence on the rate of respiration by *B. cereus*, 0.2 M sodium citrate corresponds to 0.6 M sodium chloride (Ingram, 1939b). Thus, one may compare the action of 0.2 M citrate on the growth of *E. coli* with that of 0.6 M chloride on respiration. When this is done there is a fair degree of correspondence between the two sets of data (table 3). This suggests two things: firstly, that it is legitimate to suppose that sodium citrate is roughly equivalent to three times its concentration of sodium chloride; secondly, that salts act in a similar manner on growth and on respiration.

The work described above was carried out as part of the programme of the Food Investigation Board and is published by permission of the Department of Scientific and Industrial Research.

V. SUMMARY

Manometric measurements have been made of the rate of absorption of oxygen by cells of *Bacillus cereus* and *Escherichia coli*, suspended in buffer solutions containing sodium chloride. The pH of each suspension was measured immediately after the rate of respiration had been determined.

The following conclusions may be drawn from the data:

(a) The pH of optimum respiration, about 7.3 for *E. coli* and 6.3 for *B. cereus*, is not altered when sodium chloride is added.

(b) If small concentrations of the salt are added the rate of respiration is increased; larger concentrations diminish it. This is true whatever the pH.

(c) The effect of the salt in increasing respiration is greater the more the pH differs from that of optimum respiration, be it more acid or more alkaline.

(d) The concentration of sodium chloride needed to bring about the maximum stimulation of respiration is also least at the pH of optimum respiration.

(e) The inhibition of the respiration of either organism is expressed by an equation

$$\log r = P - Q.c$$

The equation relates rate of respiration and concentration of sodium chloride whatever the pH.

(f) The values of Q in this equation are independent of pH, but differ for the two species.

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NUTRITIONAL REQUIREMENTS OF THE PNEUMOCOCCUS 1. GROWTH FACTORS FOR TYPES I, II, V, VII, VIII

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A study has been made of the nutritional requirements of the pneumococcus. The relatively simple, chemically-defined medium composed of glutathione, thiochrome, nicotinamide, betaine, flavin, glucosamine, uracil, guanylic acid, xanthine, hypoxanthine, pantothenic acid, gelatin hydrolysate, amino acids, inorganic salts, and glucose which can support the growth of the Dochez NY 5 strain of hemolytic streptococcus (Rane and Subbarow, 1938; Subbarow and Rane, 1939) was found to be deficient for pneumococci. The addition of a highly-active, purified extract of liver provided conditions suitable for good growth of certain types of *Pneumococcus*. One fraction isolated from this liver extract demonstrated the growth value of a compound similar to, and replaceable by choline. Satisfactory growth was subsequently obtained in a medium consisting of gelatin hydrolysate, certain additional amino acids, inorganic salts, glucose, choline, nicotinic acid, pantothenic acid, flavin, and thioglycollic acid. In some cases a mixture of known amino acids could be substituted for the gelatin hydrolysate.

EXPERIMENTAL

CULTURES

Pneumococci of Types I, II, V, VII and VIII have been used, one strain of each.[1] These have been passed through mice five

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[1] These strains are used regularly at the Massachusetts Antitoxin and Vaccine Laboratory in mouse protection tests for the titration of potency of therapeutic antipneumococcic serum. We wish to thank our associates at the laboratory for their kind cooperation throughout this work.

days in each week, maintaining their virulence at a point where not more than five organisms are required to kill white mice within 72 hours when injected intraperitoneally.

To provide the inocula for these experiments, cultures obtained directly from the mouse were subcultured in a meat-infusion peptone broth containing 0.1 per cent glucose, incubated for six hours at 37°C., centrifuged, washed with sterile distilled water, and resuspended in sterile distilled water. The inoculations were made in 0.1 ml. amounts. Because of the rapid autolysis that pneumococci undergo, inoculated test substances were incubated at 37°C. for only 15 to 18 hours.

Growth was measured by direct readings of turbidity with a modified Gates Nephelometer; the heavier the growth, the lower the reading. Readings ranged from 2.3 to more than 4.7, the limit of direct reading. A reading of 4.7 or more indicates little, if any growth; a reading of 3.0 or less, good growth. Controls for the different types grown in a meat infusion peptone broth, gave these readings: Type I, 2.4; Type II, 2.3; Type V, 2.5; Type VII, 2.4; Type VIII, 2.4.

The experiments described below have been repeated several times. Variations in the nature of the inoculum resulted in slight differences in readings. The figures recorded in the following tables represent values obtained from single typical experiments.

MEDIA

Basal medium (1)

Acid-hydrolyzed Eastman de-ashed gelatin	6.0	grams
d-Glutamic acid	0.1	gram
l-Cystine.	0.025	gram
KH_2PO_4..	5.0	grams
Distilled water...	800	ml.

Combined, pH adjusted with 5 N NaOH to 7.8, and tubed in 8.0 ml. amounts. Volume brought to 9.5 ml. with the addition of test substances and distilled water and autoclaved for 10 minutes at 112°C. 0.1 ml. 10 per cent $MgSO_4$ solution previously autoclaved separately for 10 minutes at 112°C., 0.1 ml. 50 per cent glucose solution also autoclaved separately, 0.1 ml. culture added to the test media. Final volume in all cases was made up to 10 ml.

The basal medium alone or combined with optimum concentrations of substances found favorable for the cultivation of the

Dochez NY 5 strain of hemolytic streptococcus did not permit growth of the strains used. However, the further addition to the latter medium of choline,[2] nicotinic acid, and thioglycollic acid produced good growth of some types even when glutathione, uracil, guanylic acid, xanthine, hypoxanthine, thiochrome, nicotinamide and glucosamine were omitted.

In preparing the final medium, the pantothenic acid,[3] nicotinic acid and choline were combined with the basal medium before autoclaving. To the sterile tubed medium were added flavin,

TABLE 1

Influence of combinations of various substances

SUBSTANCES ADDED TO BASAL MEDIUM NO. 1	NEPHELOMETER READINGS FOR TYPES:				
	I	II	V	VII	VIII
P.A.* + C. (or N.A., or T.A., or F.).. ...	—‡	—	—	—	—
P.A. + C. + N.A. (or T.A., or F.).... . ..	—	—	—	—	—
P.A. + C. + N.A. + T.A...	2.7	2.5	3.4	—	2.5
P.A. + C. N.A. + F...	—	—	—	—	—
P.A. + C. + N.A. + T.A. + F. .. .	2.4	2.2	3.0	—	2.2
P.A. + N.A. + T.A. + F......	—	—	—	—	—
Hydrolyzed P.A.† + C. + N.A. + T.A. + F.	—	—	—	—	—

* P.A. = Pantothenic acid 1 microgram/ml. medium; C. = Choline 2.5 micrograms/ml. medium; N.A. = Nicotinic acid 50 micrograms/ml. medium; T.A. = Thioglycollic acid 50 micrograms/ml. medium; F. = Flavin 0.1 microgram/ml. medium.

† Hydrolyzed by autoclaving with 0.1 N HCl for 30 minutes at 115°C.

‡ No growth.

separately autoclaved for 10 minutes at 112°C. and thioglycollic acid, previously sterilized by Berkefeld filtration. The volume was held constant at 10 ml.

Table 1 records the influence of the substances found essential for growth. With the exception of Type VII these factors in combination produced growth of all types tested; individually, the same substances were without effect. The substitution of

[2] Throughout these experiments reference is made to choline in the form of choline chloride.

[3] The pantothenic acid was assayed to be at least 50 per cent pure when compared with a preparation kindly supplied by Dr. R. J. Williams.

hydrolyzed for unhydrolyzed pantothenic acid destroyed the effectiveness of the combination.

The probability that gelatin hydrolysate includes substances still unknown made desirable its replacement by known amino acids. This medium was able to support the growth of Types II, V, and VIII but was deficient for the growth of Type I.

Basal medium (2)

d-Glutamic acid	1	gram
Glycine....	0.25	gram
l-Asparagin	0.20	gram
d-l Leucine	0.15	gram
d-Arginine carbonate................................	0.075	gram
d-l Alanine..	0.05	gram
d-l Lysine dihydrochloride............................	0.05	gram
d-l Methionine......................................	0.05	gram
l-Cystine......	0.05	gram
d-l Histidine monohydrochloride..................	0.025	gram
l-Tryptophane......	0.025	gram
β-alanine..........	0.025	gram
Nor-leucine..	0.015	gram
l-Phenylalanine......	0.01	gram
l-Oxyproline...	0.01	gram
KH_2PO_4.........................	5	grams
NaCl......................	2.5	grams
Distilled Water...........	800	ml.

Combined, pH adjusted with 5 N NaOH to 7.8, and tubed in 8.0 ml. amounts. Volume brought to 9.5 ml. with the addition of test substances and distilled water and autoclaved for 10 minutes at 112°C. 0.1 ml. 10 per cent $MgSO_4$ solution previously autoclaved separately for 10 minutes at 112°C., 0.1 ml. 50 per cent glucose solution also autoclaved separately, 0.1 ml. culture added to the test media. Final volume in all cases was made up to 10 ml.

As in basal medium (1) the pantothenic acid, nicotinic acid and choline were combined before autoclaving while sterile thioglycollic acid and flavin were added after autoclaving.

To establish the optimum concentrations of the essential growth factors, experiments were done in which certain components were varied and others, held constant. Table 2 presents the effect upon growth of different concentrations of pantothenic acid, choline and nicotinic acid when thioglycollic acid and flavin are kept constant.

It appears then that Types I, II, V and VIII have somewhat different requirements for maximum growth. A marked inter-

dependence between the three variables is evident. The optimum concentration of choline, about the same for all types tested, was somewhat limited in range. In the presence of sufficient amounts of choline and pantothenic acid, the optimum for nicotinic acid

TABLE 2

Influence of varying concentrations of pantothenic acid, choline and nicotinic acid*

NICO-TINIC ACID	CHOLINE	NEPHELOMETER READINGS FOR TYPES:									
		I			II			V		VIII	
		Pantothenic acid micrograms/ml.									
		1	0.5	0.25	1	0.5	0.25	1	0.5	1	0.5†
micrograms/ml.	*micrograms/ml.*										
50	10	2.9	3.7	>4.7	2.4	4.0	>4.7	3.8	>4.7	3.8	>4.7
50	5	2.5	2.8	2.8	2.4	2.8	>4.7	3.5	>4.7	3.2	>4.7
50	2.5	2.4	2.4	2.5	2.4	2.4	2.5	3.4	4.6	2.8	>4.7
50	1	3.4	3.3	3.4	3.3	3.5	3.6	4.7	>4.7	3.0	>4.7
50	0.5	4.2	4.3	4.5	4.0	4.1	4.6	>4.7	>4.7	>4.7	4.7
10	10	3.3	4.2	>4.7	2.5	4.0	>4.7	>4.7	>4.7	4.4	>4.7
10	5	2.7	2.9	3.0	2.3	2.8	>4.7	3.5	>4.7	4.3	>4.7
10	2.5	2.7	2.7	3.7	2.4	2.4	2.5	3.4	4.6	3.4	>4.7
10	1	3.7	3.7	3.9	3.5	3.6	3.6	>4.7	>4.7	>4.7	>4.7
10	0.5	4.4	4.6	>4.7	4.1	4.3	4.7	>4.7	>4.7	>4.7	>4.7
2	10	3.7	4.5	>4.7	2.5	4.1	>4.7	>4.7	>4.7	>4.7	>4.7
2	5	2.7	3.0	3.3	2.4	2.9	>4.7	3.5	>4.7	>4.7	>4.7
2	2.5	3.0	3.0	3.9	2.4	2.6	2.6	3.6	>4.7	3.5	>4.7
2	1	3.8	3.9	4.2	3.6	3.8	3.9	>4.7	>4.7	>4.7	>4.7
2	0.5	4.7	>4.7	>4.7	4.5	4.6	4.7	>4.7	>4.7	>4.7	>4.7

* Type I grown in basal medium (1). Types II, V, VIII grown in basal medium (2). Constant in all tubes: Thioglycollic acid 50 micrograms/ml.; flavin 0.1 microgram/ml.

† Pantothenic acid 0.25 microgram/ml. gave readings of >4.7 with Types V and VIII throughout.

was the same for all types. The optimum concentration of pantothenic acid for the types studied differed when the amounts of choline and nicotinic acid were varied.

The relationship between the concentrations of pantothenic acid and choline is well demonstrated in table 3 with Type II as

the test organism.   Increasing the amount of choline necessitated a relative increase in the concentration of the pantothenic acid to obtain equivalent growth.

The need for a sulphydryl reducing substance early became apparent.   This is not surprising in view of the importance of such an agent in the cultivation of various types of streptococci in chemically defined media.   Several compounds were tried including glutathione, ascorbic acid, and thioglycollic acid; and the latter proved to be satisfactory.   That growth does not take place in the absence of this reducing substance even in the pres-

TABLE 3

Influence of varying concentrations of choline and pantothenic acid on growth of Type II pneumococcus

CHOLINE	NEPHELOMETER READINGS PANTOTHENIC ACID MICROGRAMS/ML.						
	5	2	1	0.5	0.25	0.1	0
micrograms/ml.							
50	2.0	2.1	2.4	>4.7	>4.7	—†	—
10	2.0	2.1	2.4	3.9	>4.7	—	—
2	2.3	2.4	2.4	2.5	2.6	4.2	—
0.4	3.9	4.1	4.3	4.6	>4.7	—	—
0.08	4.3	4.6	—	—	—	—	—
0.016	4.7	—	—	—	—	—	—
0	—	—	—	—	—	—	—

* Grown in basal medium (2).   Constant in all tubes: Nicotinic acid 50 micrograms/ml.; thioglycollic acid 50 micrograms/ml.; flavin 0.1 microgram/ml.
    † No growth.

ence of optimum concentrations of the other essential factors is shown in table 4.

Flavin was not necessary for growth, but its addition increased the amount of growth slightly (table 5).

With the substitution of amino acids for gelatin hydrolysate it became possible to determine the growth values of particular amino acids.   One amino acid at a time was withheld from the medium and the effect of its omission upon the growth of the different types observed (table 6).   The question of the optimum concentration of each amino acid in the presence of the optimum concentrations of the other amino acids has not been investigated.

For the growth of Types II, V and VIII the amino acids were either indifferent, essential or inhibitory. Glutamic acid, leucine, arginine and histidine seemed to be essential for these three types.

TABLE 4

*Effect of thioglycollic acid**

THIOGLYCOLLIC ACID	NEPHELOMETER READING FOR TYPES			
	I	II	V	VIII
micrograms/ml.				
250	2.8	2.8	>4.7	>4.7
50	2.5	2.4	3.3	2.8
10	3.2	3.0	4.2	3.6
2	>4.7	>4.7	>4.7	>4.7
0.4	—†	—	—	—
0	—	—	—	—

* Type I grown in basal medium (1); Types II, V and VIII grown in basal medium (2). Constant in all tubes; Pantothenic acid 1 microgram/ml.; choline 2.5 micrograms/ml.; nicotinic acid 50 micrograms/ml.; flavin 0.1 microgram/ml.
† No growth.

TABLE 5

*Effect of flavin**

FLAVIN	NEPHELOMETER READING FOR TYPES			
	I	II	V	VIII
micrograms/ml.				
1	2.7	2.7	3.7	3.1
0.5	2.5	2.6	3.5	3.0
0.25	2.5	2.4	3.4	2.9
0.1	2.4	2.4	3.3	2.8
0.05	2.6	2.6	3.5	2.9
0.025	2.7	2.6	3.6	2.9
0.01	2.7	2.7	3.6	3.0
0	2.7	2.7	3.7	3.1

* Type I grown in basal medium (1); Types II, V and VIII grown in basal medium (2). Constant in all tubes: Pantothenic acid 1 microgram/ml.; choline 2.5 micrograms/ml.; thioglycollic acid 50 micrograms/ml.

Methionine, on the other hand, was found to be non-essential for Type II, necessary to Type V, and perhaps slightly inhibitory for Type VIII. Lysine, non-essential for Type II and inhibitory for Type V, was definitely essential for Type VIII.

The failure of Type I to grow in the amino acid medium may be due to a deficiency or to an inhibitory effect of one or more amino acids for this organism. The cultivation of this type was attempted in an amino acid medium containing substances like thiochrome, Vitamins B_1 and B_6, or the amino acids serine, threo-

TABLE 6

Amino acids and growth

AMINO ACID OMITTED FROM MIXTURE	CONCEN- TRATION	NEPHELOMETER READINGS FOR TYPES:			
		I	II	V	VIII
	mgm./ml.				
d-Glutamic acid......	1	—†	—	—	—
Glycine........................	0.25	>4.7	2.3	>4.7	3.5
l-Asparagin...................	0.2	>4.7	2.8	>4.7	>4.7
d-l Leucine...................	0.15	>4.7	>4.7	>4.7	>4.7
d-Arginine carbonate..........	0.075	—	—	—	—
d-l Alanine.....	0.05	>4.7	2.3	3.3	2.6
d-l Lysine dihydrochloride.....	0.05	>4.7	2.3	2.9	>4.7
d-l Methionine......	0.05	>4.7	2.3	4.7	2.5
l-Cystine.............. . .	0.05	>4.7	2.3	3.8	>4.7
d-l Histidine......	0.025	—	—	—	—
l-Tryptophane............	0.025	>4.7	2.8	3.2	2.7
β-alanine.	0.025	>4.7	2.4	2.9	—
Nor-leucine......	0.015	>4.7	2.3	2.9	2.5
l-Phenylalanine.... . . .	0.01	>4.7	2.8	3.4	2.8
l-Oxyproline	0.01	>4.7	2.3	3.4	2.8
Control. None omitted .	—	>4.7	2.3	3.4	2.8
Control. All omitted.... .	—	—	—	—	—

* Constant in all tubes: Pantothenic acid 1 microgram/ml.; choline 2.5 micrograms/ml.; nicotinic acid 50 micrograms/ml.; thioglycollic acid 50 micrograms/ml.; flavin 0.1 microgram/ml.

† No growth.

nine, valine, iso-leucine, proline and tyrosine but in every case without success.

Media found adequate for Types I, II, V and VIII were deficient for Type VII. The addition of other substances such as Vitamins B_1 and B_6, thiochrome, uracil, guanylic acid, adenylic acid, inosinic acid, carnitine, compounds related to nicotinic acid, cadaverine, spermin, putrescine, pimelic acid, quinic acid, divicine, ergothionine, xanthine, hypoxanthine and others also

failed to induce the growth of this type. One strain, obtained from Dr. M. Finland, grew scantily in the regular amino acid medium.

Preliminary experiments done with one strain each of other types of pneumococci[4] gave readings ranging from 3.0 to 4.1 for Types VI, IX, XII, XIV, XXI, XXIV, and XXX grown in the amino acid medium.

DISCUSSION AND SUMMARY

Highly virulent strains of Types I, II, V and VIII pneumococci have been grown in a medium consisting of gelatin hydrolysate, certain additional amino acids, inorganic salts, glucose, choline, nicotinic acid, pantothenic acid and thioglycollic acid. Though in general comparable, both qualitative and quantitative differences were encountered in the essential factors for each type.

A mixture of known amino acids may replace the gelatin hydrolysate in media for Types II, V and VIII but will not support the growth of Type I. However, each type exhibited distinct amino acid requirements; an amino acid necessary for the growth of one type sometimes proved to be inhibitory or indifferent for another.

No growth was observed with Type VII in either medium even with the inclusion of such other compounds as pyridine derivatives, purines, pyrimidines, Vitamins B_1 and B_6 and thiochrome.

The addition of flavin gave slightly increased growth but was not essential.

Possible differences of growth requirements of strains within a type were not studied.

Note: Williams and Major (1940) published their paper on the structure of pantothenic acid about the time our experiments were concluded. Since then a preparation of synthetic pantothenic acid prepared in our laboratory from β-alanine and the acid component reported by Williams and Major has been found to replace the natural compound in the cultivation of Type II, the

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[4] We wish to thank Dr. Finland for these organisms.

only type tested at this point. It follows that Type II at least may be grown in a medium chemically defined except for possible impurities in natural amino acids.

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STUDIES ON THE SPIRILLEAE: METHODS OF ISOLATION AND IDENTIFICATION[1]

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In the recent literature of bacteriology scant attention has been paid to the genus *Spirillum* Ehrenberg. Many of the textbooks treat this as an obscure group. Unfortunately, the most recent edition of Bergey's Manual (1939) is inadequate in its coverage of the genus, in spite of recent corrections. No more than a citation has been given to the one recent general work on the subject, a thesis by Giesberger (1936).

Two difficulties have obscured our knowledge of the spirilla. Their isolation is difficult by the usual bacteriological techniques. And their screw-like morphology, which allows four descriptive measurements, has led to the naming of many species without supporting cultural characteristics. Some forty species are recorded in the literature; probably less than ten of these could be recognized with surety from their original descriptions.

This paper presents a description of techniques of isolation which are comparatively simple and effective, at least for some species, and a characterization of four spirilla and two vibrios thus obtained from stagnant waters of Montana and Minnesota.

METHODS OF ISOLATION AND CULTURE

Since the time of Kutscher (1895) the preparation of an infusion has been the first step in the isolation of spirilla. This is easily

done with liquid samples such as stagnant water, sewage, and liquid dung considered in the present study.   In practice, samples are gathered in wide-mouth jars and brought to the laboratory. A small amount of organic matter such as peptone, salts of organic acids, lettuce leaves, lean meat, or fish is added.   The last has proved particularly suitable.   After one or two days incubation, motile spiral forms may be microscopically observed in a hanging drop preparation from the extensive flora which develops.   If a number of samples are studied, perhaps one or two will show at some time a comparatively high proportion of one or more spiral forms.   In the early stages of this study repeated attempts were made to plate out onto beef-peptone agar from such infusions which looked to be teeming with spirilla.   Various other methods and media were used without success until the procedure outlined below was tried.

An infusion containing large numbers of spirilla is sterilized by filtration through a Berkefeld filter, portioned out aseptically into a number of sterile petri dishes, and hardened by the addition of sterile 2 per cent agar solution.   A rough separation of motile from non-motile forms is made from a hanging drop of another infusion containing large numbers of spirilla by the following method.   A glass tube is drawn out to a long capillary of fairly uniform bore.   Sterile water or infusion liquid is drawn up in this tube to a length of about 2 or 5 cm.   Two marks are made on a piece of paper to indicate the length of the water column.   From the hanging drop selected about 2 to 5 cm. more are drawn up into the capillary.   This is allowed to lie quietly in a horizontal position for about half an hour.   The end of the capillary, up to a point somewhat above the original infusion-sterile liquid junction, is cut off with a knife.   The remaining liquid is blown out into a test tube of the previously sterilized infusion liquid.   This procedure effects a concentration of spirilla, since they are generally the most actively motile of the bacteria present.   While not always necessary, it tends to cut down the number of non-motile forms in some infusions.

The method of plating has been adapted from the single spore technique described by Kauffman (1908).   Three or four serial

1:10 dilutions are made into tubes of the sterilized infusion liquid from a tube inoculated with a loopful of infusion, or with the liquid from the capillary containing the "concentrated" spirilla. Capillary pipettes are drawn out from pieces of glass tubing in such a way that water blown out of them emerges in the form of very small droplets. Liquid drawn up from each of the dilutions into these sterilized pipettes is then blown out on to the agar surface of one of the petri plates, so as to give a fairly uniform distribution of droplets. When the appropriate dilution is used, the average droplet contains one or less than one organism. This organism is well isolated on the agar surface and yet immersed in a liquid medium for the first several hours. Such an arrangement is particularly favorable for the spirilla, which generally seem to prefer liquid media.

All bacteria develop slowly on the infusion agar, which is rather poor in nutrients. Generally, a week's incubation at room temperature is required. Unfortunately, in spite of all of the above precautions, there is usually only a small percentage of colonies which contain spiral forms, and the author has found no general characteristic by which the colonies of all spirilla may be recognized. For two large species, *Spirillum undula* and *Spirillum serpens*, individual cells protruding from the colony edge may be recognized under the 16 mm. objective. For smaller species the colonies are indistinguishable from those of many other forms. However, all species so far obtained have come from small, more or less colorless, translucent or transparent colonies; never from large or spreading or highly colored colonies. Colonies from which spirilla can be demonstrated in a hanging drop are transferred to tubes of the infusion liquid previously sterilized by filtration. A second plating-out has seemed unnecessary, since the original colonies are well isolated. Any mixed culture is quickly observed, for contaminants almost invariably grow much faster than the spirilla. From the culture in the sterilized infusion media, transfers are made to the usual beef-peptone broth and subsequently to agar slants.

Several modifications of the above method have been tried. A more easily prepared, and nearly as satisfactory, medium for the

isolations is a fish broth, liquid or solidified with agar. About 10–20 grams of fresh fish are simmered in a liter of tap water over a slow flame for several hours. This is filtered, the pH adjusted to 7.0, agar added if desired, tubed and autoclaved. During the sterilization the pH usually rises to about 7.2–7.4.

The continued cultivation of spirilla on the usual beef-peptone agar is not difficult, provided several precautions are taken. Although no conclusive tests have been made, it has seemed advantageous to dissolve the peptone and beef extract by 20 minutes autoclaving before the pH adjustment is made. For growth on agar heavy inoculations are necessary and should be made into the condensation water of a fresh slant. The liquid is then drawn up over the slant with the inoculating loop. Observing these precautions, some 60 strains have been carried in stock culture without difficulty by transferring once a month.

During the first part of this investigation about 20 strains of spiral organisms were isolated from surface waters near Bozeman, Montana. These were studied in the usual way in regard to their morphology and cultural characteristics. At about this time there came to the author's attention the thesis of Giesberger (1936) describing five species of spirilla isolated by him in Holland. His method of isolation consisted of repeated streaking out and the use of a micro-manipulator, since only very small colonies were produced under his conditions. Some 60 strains were thus obtained for study. Of those which could be kept in culture he was able to obtain a clear-cut differentiation into four groups on the basis of their utilization of various carbon sources. These groups corresponded exactly with the four different types of morphology found. By comparison with previously described forms these were named *Spirillum serpens, S. tenue, S. itersonii,* and *S. undula.* A fifth species obtained from horse manure, but which could not be cultured so readily, was described as *S. kutscheri.*

Unfortunately my cultures were accidentally lost before Giesberger's methods of study could also be applied. A second set of cultures isolated in 1936 were also lost before they could be adequately studied.

Early in 1939 a third series of cultures was obtained from various sources near Minneapolis, Minnesota. Over a hundred cultures were isolated and carried along in duplicate. Of these, many have been subsequently discarded due to contamination, apparent change in morphology, or to the fact that they seemed to be borderline cases with cells only slightly curved, or with only a small percentage of cells curved. From this experience it seems likely that there may be numerous borderline microörganisms lying between the spirilla and vibrios on one hand and the *Pseudomonas*-type rods on the other. A consideration of such borderline organisms is beyond the scope of the present study, since even the distinct spirilla are still so incompletely known. A few attempts at isolation from barnyard manure were also made, but with little success. The one culture obtained grew poorly on all the media tried and soon died. Giesberger's suggestion (p. 57) that the dung-spirilla belong to species distinctly different from those found in the usual surface waters may indeed be correct.

STUDY OF THE ISOLATED STRAINS

Of the 107 strains originally isolated and cultured in duplicate, 54 which continued to show a typical spiral morphology were retained for study. Previous work on the strains isolated in Montana had indicated temperature optima in the vicinity of 32°C. and pH optima of about 7.2–7.4. This is entirely in accord with the results of Giesberger. Because of the large number of cultures, they were incubated at room temperature (24–30°C.).

The method of differentiation used by Giesberger was closely followed, except that of the 29 carbon sources which he used six were selected which showed clear-cut differences between his various species. To the inorganic medium containing 0.05 per cent $MgSO_4$, 0.05 per cent K_2HPO_4, and 0.05 per cent $CaCl_2$ there was added a nitrogen source (0.1 per cent) and a carbon source (0.5 or 1.0 per cent). The pH was adjusted to 7.0 to 7.2 against the bubbling hydrogen electrode and the media sterilized by filtration through a Seitz filter. (Autoclaving induces precipitation and attendant pH changes.) Tubes were inoculated

with a loopful of a young broth culture. The small additional organic matter thus introduced in the inoculum was apparently too small to influence the results, since all cultures showed zero growth on at least one medium.

With 1.0 per cent calcium lactate as the carbon source, four nitrogen compounds were tried: NH_4Cl, asparagin, KNO_2, KNO_3. As shown below, NH_4Cl gave at least fair growth with nearly all strains and was therefore used in the series of various carbon sources. Glucose, fructose, glycerol, ethyl alcohol, calcium pyruvate, and calcium lactate were studied as sources of carbon. The last two were used in a concentration of 1.0 per cent and without any $CaCl_2$ in the media.

The tubes were examined after seven days incubation at room temperature. Growth was roughly estimated by the degree of turbidity. Results are presented in the summarized descriptions below. A supplementary microscopic examination was made of all tubes showing even the slightest turbidity. This revealed surprising changes and irregularities, both in size and in shape. For instance, on calcium lactate *S. serpens* showed numerous cells in which one end had enlarged into a distinct ball, sometimes comprising a half of the total cell material. And all three strains of *Vibrio* #2 (see below) on several of the synthetic media showed only round or oval cells, though subsequent inoculation to peptone-beef broth always brought back the original curved form. It is apparent that valid cell measurements in these genera can be made only under standard and reproducible conditions.

Microscopic examination of cells in a hanging drop preparation was made on 12–18 hour broth cultures (30°C.) for all strains. This allowed a division of the strains into six groups. Such a morphological grouping showed good correlation with the data on the utilization of the various sources of carbon. In general, the amount of growth on any one synthetic medium was the same for all strains of a morphological group. Exceptions are noted.

Flagellation was studied by staining the cells from a 12–24 hour agar slant culture by the method of Gray (1926). In the author's hands this method, with minor modifications, has given consistently good results with spirilla for the past five years. Charac-

teristics of growth on various routine culture media have been determined by the usual methods. Summarized morphological and cultural descriptions of six of the organisms isolated are presented below. (Characteristics significantly different from those described by Giesberger are marked with an asterisk.)

Spirillum I, Spirillum serpens (Müller) Winter

Strains studied: 18, 20, 64, 66, 67, 70, 82, 83.

Cells motile by means of bipolar bundles of flagella. Cytoplasm often highly granular. Cell thickness: 0.8–1.0 μ; cell length: 10–25 μ; width of spiral: 1.5–2.0 μ; wave length: 7.5–9.0 μ.

Gelatin not liquefied.*

Agar colonies: circular, rough, translucent, the edge irregular; flat, the center characteristically raised and radiately ridged.

Agar slant: beaded, gray, glistening.

Broth: scant, turbid growth.

Litmus milk: unchanged.

Potato: no growth.*

Neither nitrites nor gas produced from nitrates.

Indole not produced.

H_2S formed slowly by most strains.

Aerobic (growth only in upper 3 mm. of tube shake culture).

Optimum temperature: about 32°C.

Utilized as sources of nitrogen: NH_4Cl (except strains 18, 20), asparagin; as sources of carbon: lactate (except strains 18, 20), pyruvate.

Isolated from greenhouse pool, sewage effluent, Mississippi River, lake water, stagnant ditch water.

Spirillum II, Spirillum itersonii Giesberger

Strains studied: 11, 12, 13, 15, 16, 17, 78, 80, 81, 84.

Cells motile by means of bipolar bundles of flagella. Cell thickness: .5 μ; cell length: 4–8 μ; width of spiral: 1.2–1.8 μ; wave length: 3.0–3.5 μ.

Gelatin not liquefied.

Agar colonies: circular, smooth, glistening, entire, convex.

Agar slant: filiform, glistening, gray.

Broth: strong, turbid growth.

Litmus milk: becoming slightly alkaline.

Potato: moist brown growth.

Slight production of nitrites from nitrates on solid media, none in broth; no gas.

Indole not produced.

H₂S produced.

Aerobic (growth only in upper 3 mm. of tube shake culture).

Optimum temperature: about 32°C.

Utilized as sources of nitrogen: NH₄Cl, asparagin; as sources of carbon: lactate, pyruvate, glucose, fructose, glycerol, ethyl alcohol.

Isolated from greenhouse pool, Mississippi River.

Spirillum III

Strains studied: 14, 37, 38, 39, 40, 41, 42, 45, 46, 47.

Cells motile by means of bipolar bundles of up to six flagella, somewhat attenuated at one or both ends. Cytoplasm highly granular. Cell thickness: 0.7–0.8 μ; cell length: 2.5–5.0 μ; width of spiral: 1.2–1.5 μ; wavelength: 2–3 μ.

Gelatin not liquefied.

Agar colonies: circular, smooth, entire, convex, translucent.

Agar slant: filiform, glistening, gray.

Broth: strong clouding, turbid sediment.

Litmus milk: becoming slightly alkaline.

Potato: brown growth.

Nitrites and gas produced from nitrates.

Indole not produced.

H₂S produced.

Aerobic (growth only in upper 3 mm. of tube shake culture).

Optimum temperature: about 37°C.

Utilized as sources of nitrogen: NH₄Cl, asparagin; as sources of carbon: lactate, pyruvate, glucose, fructose, ethyl alcohol.

Isolated from: greenhouse pool, stagnant ditch water.

Spirillum IV, Spirillum undula (Müller) Ehrenberg

Strains studied: 3, 5, 6, 8, 10.

Cells widely-coiled, motile by means of bipolar bundles of flagella. Cytoplasm often highly granular. Cell thickness: 0.9–1.2 μ; cell length: 8–16 μ; width of spiral: 3.0–3.5 μ; wavelength: 6–8 μ.

Gelatin not liquefied.

Agar colonies: circular, smooth, entire, convex, translucent.

Agar slant: beaded-filiform, glistening, gray.

Broth: slight granular growth.

Litmus milk: growth none or very slight; medium unchanged.

Potato: no growth.*

Neither nitrites nor gas produced from nitrates.

Indole not produced.

H₂S not produced.

Aerobic (growth only in upper 15 mm. of tube shake culture).

Optimum temperature: about 32°C.

Utilized as sources of nitrogen: NH₄Cl, asparagin; as sources of carbon: lactate, pyruvate (* not ethyl alcohol as listed by Giesberger).

Isolated from greenhouse pool, stagnant ditch water.

Vibrio #1

Strains studied: 27, 56, 57, 61.

Cells of seldom more than a single arc, motile by means of one or (rarely) two polar flagella.   Cell width: 0.8 μ; cell length: 2.5–4.0 μ.

Gelatin not liquefied.

Agar colonies: circular, smooth, entire, convex, translucent.

Agar slant: filiform, gray growth; a characteristic dark brown pigment diffuses slowly down into the agar.

Broth: clouding scant; medium darkened in time.

Litmus milk: becoming slightly acid.

Potato: no growth.

Neither nitrites nor gas produced from nitrates.

Indole not formed.

H₂S not produced.

Aerobic (growth only in upper 3 mm. of tube shake culture).

Utilized as sources of nitrogen: NH₄Cl (except strain 27); as sources of carbon: lactate (except strain 27), pyruvate, fructose.

Isolated from greenhouse pool, liquid entrapped in a pitcher plant.

Vibrio #2

Strains studied: 29, 36, 48.

Cells seldom of more than a single arc, motile by means of one or (rarely) two polar flagella.   Cell width: 1.1–1.3 μ; cell length: 3-5 μ.

Gelatin not liquefied.

Agar colonies: circular, smooth, entire, flat, translucent.

Agar slant: filiform, glistening, gray.

Broth: scant clouding; often a surface membrane.

Litmus milk: becoming slightly alkaline.

Potato: brown growth.

Nitrites and gas produced from nitrates.

Indole not produced.

H₂S produced.

Aerobic (growth only in upper 3 mm. of tube shake culture).

Utilized as sources of nitrogen: NH₄Cl; as sources of carbon: lactate, ethyl alcohol (except also slight growth in fructose by strain 36 and in glycerol by strain 29).

Isolated from greenhouse pool.

The six forms described above have been designated *Spirillum* or *Vibrio* on the basis of the two coincident characteristics by which these two genera are commonly distinguished [cf. Bergey (1939), Migula (1900), Lehmann and Neumann (1927)]. The genus *Spirillum* is characterized by rigid, truly spiral cells, motile by means of polar bundles of flagella. The genus *Vibrio* (or *Microspira* according to Migula) is distinguished by the occurrence of rigid, curved cells of only a single arc (comma-form) and with only one to three polar flagella at one or both ends.

These characteristics are admittedly poor for the separation of the two genera. Older cultures of a *Vibrio* often show a high percentage of cells in definite spirals, while very young cultures of a *Spirillum* contain a large proportion of cells of only a single turn. On the other hand the difficulties of staining flagella are well known. If inadequately stained, the compact flagella-bundle of a distinct *Spirillum* such as *S. undula* may easily be mistaken for a single flagellum. The difficulty of relying on flagellation is illustrated by the case of *Spirillum virginianum*. This was originally described by Dimitroff (1926) as possessing a single polar flagellum. However, cultures of the same strain obtained from the American Type Culture Collection by Giesberger (1936) and by the author have shown polar bundles of flagella.

The author therefore takes the view that until some clear-cut case to the contrary is discovered, the separation of the genera *Vibrio* and *Spirillum* must be based upon flagellation and spiral length as coincident characteristics, however inadequate these may be.

Most of the 57 strains isolated can be placed in six groups, of which four are definitely spirilla and two are vibrios. Several of the strains were lost or became contaminated and were discarded.

And several others showed distinct differences in morphology and cultural characteristics from all of the six listed above. These strains will not be considered in the present paper since no two of them were sufficiently alike to give duplicates for study.

The literature on the genus *Spirillum* has been carefully studied in order to verify Giesberger's nomenclature and to aid in the identification of the species described above. All of the literature thus reviewed is listed in the bibliography, for the most part without specific citation in the text. A common characteristic of the older descriptions is their inadequacy. Whether or not a newly isolated culture should be identified with one of the species described earlier is at best a delicate problem. In this regard the author recognizes the validity of the species described by Giesberger and follows the principle laid down by him (p. 56):

"schien es mir dennoch emphehlenswert, eine neue Systematik der Gattung *Spirillum* in möglichst engem Anschluss an die Ergebnisse früherer Untersucher durchzuführen und wo dies nur möglich schien, die alten allgemein angewandten Artnamen beizubehalten."

The genus *Vibrio* has been considered only incidentally, since methods of isolation and study were directed principally toward the genus *Spirillum*. Of the two vibrios isolated, *Vibrio* #1 corresponds to *Vibrio nigricans* Weibel (Cf. Migula (1900) p. 1013), while *Vibrio* #2 seems to be a previously undescribed form.

Of the four species of *Spirillum* described above, three agree quite well with the descriptions of *S. serpens*,[3] *S. itersonii*,[3] and *S. undula* given by Giesberger. These names have therefore been applied to the corresponding species descriptions. A fourth species, *Spirillum* III,[3] is clearly distinct from any of those described by Giesberger. This organism is clearly a *Spirillum* rather than a *Vibrio*, since in broth cultures observed during the logarithmic phase of growth (18 hours at 30°C.) most of the cells are of more than a single arc, and since flagella stains show many cells with six or eight flagella in each bundle. It differs from *S.*

[3] Cultures of these organisms have been placed with the American Type Culture Collection, Georgetown University, Washington, D. C.

tenue in its ability to utilize ethyl alcohol and in the wave length of its spiral. It could never be considered a tenuous organism, and in fact was originally tentatively described in our records as "short and fat." Its inability to liquefy gelatin and its short wave length clearly set it apart from *S. virginianum*.

The descriptions of nearly 40 spirilla recorded in the literature have been examined without finding any with which *Spirillum* III may be surely identified. In a number of respects it is similar to *Spirillum lipoferum* of Beijerinck (1925) and Schröder (1932). This organism was originally isolated from enrichment cultures of *Azotobacter*. It is described as a short, plump, highly granulated *Spirillum*, motile by means of polar flagella-bundles, varying widely in cell size and proportions, and often containing fat globules. Both Beijerinck and Schröder were chiefly interested in the question of nitrogen fixation by this form and neglected to make any complete characterization. They made no mention of cell dimensions. The presence of fat globules is hardly distinctive since it is shown by other spirilla. Giesberger (1936, p. 24) refused to recognize this as an adequately characterized *Spirillum*. Following Beijerinck's directions, he isolated several strains of a similar organism from garden earth and canal water. These cultures contained cells with generally only a single turn and with monotrichic flagellation. He concluded that the organism was not a true *Spirillum*.

Unfortunately Giesberger's experience does not clarify the situation, since there is no way of establishing the identity of the organisms observed by Beijerinck and by Giesberger. Both Beijerinck and Schröder were quite specific in describing the lophotrichic flagellation. And in fact *Vibrio* #2 (see above) could be identified with Beijerinck's description in nearly every respect except that of flagellation.

It is apparent that at present *Spirillum* III can neither be positively identified with, nor differentiated from, the organism described by Beijerinck as *Spirillum lipoferum*. And under these conditions it seems better to leave the organism described in this way than to add what might be merely another name to the already long list of species of *Spirillum*.

SUMMARY

1. A method of isolation has been described which is particularly adapted to a number of species of *Vibrio* and *Spirillum*. The essential feature of this technique is that agar plate colonies are initiated from cells planted in droplets of liquid media scattered over the agar surface. After several transfers on sterilized infusion media most cultures can be carried on the usual agar slants.

2. Four species of *Spirillum* and two of *Vibrio* have been studied in regard to their morphology, routine culture characteristics, and utilization of various carbon and nitrogen sources in synthetic media. After a critical study of the literature, three of the spirilla have been identified with the previously described species, *S. undula*, *S. itersonii*, and *S. serpens*. A fourth, similar in certain respects to *S. lipoferum* of Beijerinck but not definitely the same, has been described but not named.

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PLATE 1

FLAGELLA STAINS

FIG. 1. *Spirillum* I (*S. serpens*). 1000×.

FIG. 2. *Spirillum* II (*S. itersonii*). 1000×.

FIG. 3a. *Spirillum* III. 1000×.

FIG. 3b. *Spirillum* III. 1700×.

FIG. 4. *Spirillum* IV (*S. undula*). 1000×.

FIG. 5. *Vibrio* #1. 1000×.

FIG. 6. Undetermined *Spirillum* isolated from stagnant water in Montana. 1000×.

fig. 1
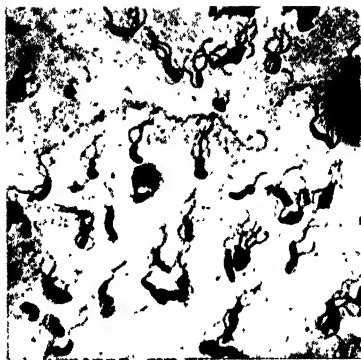
fig. 2
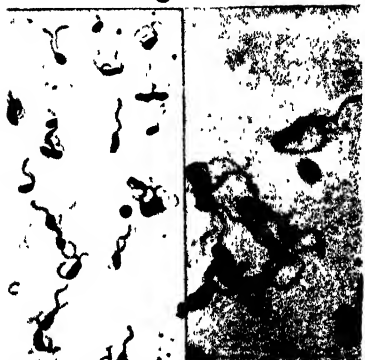
fig. 3a   fig. 3b

fig. 4
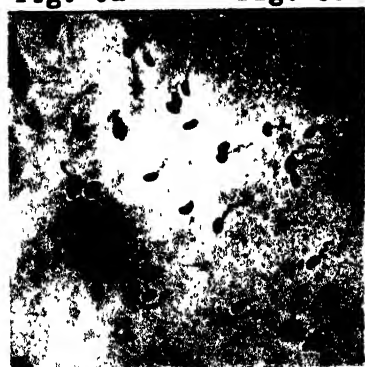
fig. 5
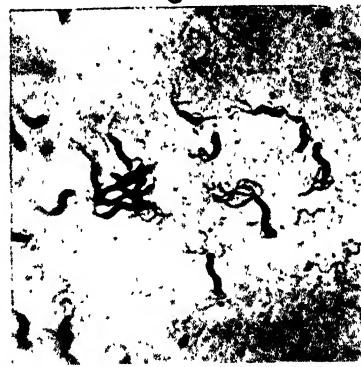
fig. 6

(Jack Myers: Studies on Spirilleae)

RELATION OF PROTEOLYTIC ENZYMES TO PHASE OF LIFE CYCLE OF BACILLUS LARVAE, AND TWO NEW CULTURE MEDIA FOR THIS ORGANISM

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INTRODUCTION

In the several descriptions of *Bacillus larvae* (White), the cause of American foulbrood of bees, that have appeared in the literature, there are discrepant findings concerning the ability of the organism to produce proteolytic enzymes. The work here reported, a continuation of a study made earlier by Sturtevant (1924), reveals that the elaboration of certain proteolytic enzymes by the bacillus is intimately related to its life cycle. This fact may help to explain some of the apparently divergent earlier results.

REVIEW OF LITERATURE

The gelatin-liquefying property of *Bacillus larvae* was not determined by White (1906), since, as he says, the organism fails to grow at the temperature at which gelatin remains solid. Maassen (1908), working at the same time, reported that a slow but gradual liquefaction took place unless inhibited by the presence of glucose in the medium. Sturtevant (1924) showed that protein decomposition as a result of the growth of *B. larvae* could be demonstrated, both in infected bee larvae and in egg-yolk broth, by chemical analyses, but direct action of the organism upon gelatin was not sufficient to be called definitely positive,

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[1] A contribution from the Bureau of Entomology and Plant Quarantine, United States Department of Agriculture, in cooperation with the University of Wyoming.

although infected ropy larval material did produce rapid gelatin liquefaction. Lochhead (1928) found gelatin liquefaction to be variable, depending upon the strain of the organism used. In litmus milk he observed the formation of a rennet-type curd, without any peptonization, even after prolonged incubation. Stoilowa (1938) reported that her cultures of *B. larvae* liquefied gelatin and coagulated milk without hydrolysis.

Since *Bacillus larvae* fails to grow on the ordinary laboratory media, complex media have been devised for its cultivation, some of which are both difficult and tedious to prepare, as well as requiring materials not generally available. White (1906) first succeeded in growing *B. larvae* on a honeybee-larvae-infusion-agar. He later used sterile unheated brood filtrate in place of infusion (1907), and eventually he found that sterile unheated egg yolk added to the base medium (1919) could be substituted for the larval material. Sturtevant (1924) devised a medium in which sterile egg yolk was added to a yeast-peptone base. Later he added carrot extract (1930) to this medium and was able to obtain growth with a minimum inoculum of 50,000 spores per milliliter (1932). Lochhead (1928) found that when carrot extract is present in the medium an apparently specific nitrite test for *B. larvae* can be obtained. More recently Tarr (1937a, 1937b, 1938), working at Rothamsted, has utilized modifications of the larval-filtrate media, as well as a minced egg-embryo medium. The latter substrate enabled him to obtain growth, usually with inocula containing as few as 1,000 spores, and occasionally with approximately 140 or 100 spores. He used a larval-filtrate medium for the production of a spore crop with vegetative cultures. Stoilowa (1938) reported good growth on a glucose-blood agar.

EXPERIMENTS WITH DISEASED AND HEALTHY HONEYBEE LARVAE

When litmus milk was tested for suitability as a medium for the cultivation of *Bacillus larvae* from "scales,"[2] a surprising

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[2] A diseased dead honeybee larva which dries down and adheres to the base of the brood cell is known to beekeepers as a scale. Each scale carries on an average approximately 2,500,000,000 spores.

reaction was noted; peptonization became apparent after only a few hours of incubation at 37°C., and was usually complete within 12 to 18 hours.   Since vegetative cultures of *B. larvae* inoculated into milk produce only an acid reaction with reduction and curdling, it appeared that the proteolytic enzyme must have been contained in the scale prior to inoculation and be independent of any germination and growth following inoculation.

That this was indeed true was shown in several ways.   First, a sterile, unheated filtrate of a scale suspension in water produced both peptonization of litmus milk and hydrolysis of gelatin; a similar reaction could be obtained with unheated and unfiltered suspensions in the presence of chloroform.   Again, the same suspension peptonized milk and liquefied gelatin at 50°C., which is above the maximum temperature for the growth of *B. larvae*. Finally, the suspension was inactivated by heating to 93°C. for 20 minutes, although the spores subsequently germinated and reproduced.

To determine whether enzymes were produced by healthy honeybee larvae, the bacillus, or an interaction between the two, a series of healthy and diseased larvae were inoculated into litmus milk.   It was found that unsealed larvae, which at this stage are constantly fed honey and pollen by the nurse bees, contained some proteolytic enzymes, possibly derived from the pollen content of the food.   After the larvae were sealed over in the cells, being in the prepupal stage and no longer feeding, these enzymes disappeared in healthy individuals.   On the contrary, diseased larvae, after being sealed, showed a much higher enzyme content as the symptoms of the disease increased.   Also, as the severity of the disease increased, as indicated by decomposition, the percentage of spores compared with vegetative cells increased.

DEVELOPMENT OF NEW CULTURE MEDIA FOR BACILLUS LARVAE

For the study of pure cultures of *Bacillus larvae* and for use in the study of the production of enzymes on a solid substrate, two relatively simple media recently have been devised in the Intermountain States Bee Culture Field Laboratory at Laramie, Wyo., one for diagnostic purposes and the other for stock culture studies.

These media have the following advantages over those previously used: (a) Growth is produced with an inoculum of theoretically only one spore, as calculated from the dilution of a standard suspension; (b) it is readily prepared without special technique or materials; and (c) it is clear and transparent. The composition of the diagnostic medium is as follows:

Glucose..	10 gram
Bacto neopeptone	10 gram
Bacto yeast extract......	10 gram
Agar.....	15 gram
Carrot extract	200 ml.
Cysteine	80 mgm.
Distilled water..........	800 ml.

(Adjust to pH 6.8–7.0)

The nitrite test for *Bacillus larvae*, which, together with the microscopic appearance of the culture, appears to be highly reliable (Lochhead (1928), Sturtevant (1932), Hitchcock (1936)), also is obtained with this medium after a few days' growth. The lag phase before growth appears is markedly reduced compared with the egg-yolk medium, and sensitivity is greater. In fact, with one strain of *B. larvae* no growth was initiated with an inoculum of less than 50 million spores per milliliter on the egg-yolk agar, even after 30 days' incubation, whereas the new medium produced growth after 4 days with an inoculum of one or only a few spores.

The method of inoculation was that previously used by Sturtevant (1932). By this method the spores are suspended in sterile distilled water, and the number of spores per milliliter is determined by direct count under the microscope. From this standard suspension appropriate dilutions are made to obtain the desired number of spores per milliliter for inoculation of media. One milliliter of the dilution to be cultured is added to the slanted medium in a tube, which is then incubated at 37°C. in an upright position. It is to be pointed out that growths on the new medium with such small inocula are obtained only when the cells are added to the slant, in this case suspended in 1 ml. of sterile water, and the same results are not obtained even when much larger numbers of spores are added by the loop-inoculation method. In the

diseased larvae available for diagnosis and culture, the organism is found, almost invariably, only in the spore stage and generally in pure culture; hence the importance of obtaining spore germination as well as vegetative growth.

While the method described above is useful in routine diagnosis, it has a serious disadvantage as a stock medium. Although a large amount of vegetative growth occurs, few or no spores are formed, and the culture often dies out after a few weeks, the vegetative rods disintegrating. In a series of experiments it was found that whenever carrot extract was present in the medium sporulation was suppressed or inhibited, but when the carrot extract was omitted and neopeptone was present sporulation freely occurred. These data were obtained on vigorous strains of *B. larvae* capable of sporulation.

When the concentrations of the ingredients of the medium, without the carrot extract, were varied in order to approximate the optimum concentrations for growth and sporulation, it was found that the percentage of sporulation was highest in the medium made up to full strength, and such medium also gave the greatest total growth and consequently the greatest total spore harvest. This is contrary to the findings of Tarr (1937a).

The medium finally adopted for stock culturing, as well as for the production of spores for experimental purposes, is given below:

Glucose	10 gram
Bacto neopeptone.. ..	10 gram
Bacto yeast extract	10 gram
Cysteine	80 mgm.
Agar	15 gram
Distilled water	1000 ml.

(Adjust to pH 6.8-7.0)

With this medium there is a slightly longer lag phase than with the carrot-extract type, and the nitrite test cannot be obtained on it, but it has been found very satisfactory for its particular use.

EXPERIMENTS WITH SPORES OF BACILLUS LARVAE ON NEW CULTURE MEDIUM

In order to ascertain whether a correlation similar to that described in diseased larvae existed between enzyme content and

phase of life cycle of *Bacillus larvae, in vitro*, to each tube of a series of slant cultures of various ages growing on the new stock medium 10 ml. of sterile litmus milk was added, and the whole incubated at 37°C. The results exactly paralleled those obtained with diseased larvae; that is, with young cultures not yet sporulating no peptonization occurred, but as sporulation began some peptonization could be noted, and older cultures which had completely sporulated gave a strong reaction after a few hours' incubation. Analogous results were obtained with respect to gelatin liquefaction. From this it would seem that these enzymes are released as the old cells disintegrate to free the newly formed spores. However, a parallel experiment with several apparently asporogenous strains of *B. larvae*, derived from sectors of giant colonies, showed that mere autolysis of vegetative cells without attendant sporulation engenders the production of no such enzymes. The appearance of these enzymes appears to be peculiar to the sporulation process.

It also appeared possible that these proteolytic enzymes were produced by the activity of the so-called dormant spores. Such activity by dormant spores has been claimed by Effront (1917), Reuhle (1923), Cook (1931), and Tarr (1934). The theory of Effront, that under unfavorable conditions dormant spores elaborate extra-cellular enzymes and are "the more productive of enzymes the more difficult their germination," was first examined.

A spore suspension was prepared, and a portion removed before heating to serve as a control. The remainder was heated in flowing steam for 15 minutes at 93.5°C., which inactivates the enzymes but does not notably affect spore viability. One-milliliter amounts of the control and of the heated suspension were placed in 5 ml. of sterile litmus milk and incubated at 37°C. Three 5-ml. amounts of heated suspension were incubated at 37°C. for 1 week, one without any further treatment, one with a small amount of toluene, and the third with a small amount of chloroform, and then tested by inoculation into litmus milk and incubation at 37°C. The results of this experiment, which was repeated with the same results, are given in table 1.

Here there are no indications of enzymatic activity by dormant

spores, nor did the presence of antiseptics induce such activity. Had such antiseptics stimulated enzyme elaboration, it seems reasonable that this would have been detected, inasmuch as the unheated control kept 1 week over chloroform did not lose its potency.

The possibility that the heating had destroyed the capacity of the bacterial protoplasm to form enzymes was examined.   The spores from a heat-inactivated suspension were cultured on the stock medium, and, after growth and sporulation had occurred, a suspension of these spores proved to be active again in hydrolyzing litmus milk.   Such heating, then, seemed to have no permanent effect on the culture.

TABLE 1

Peptonization of spore suspensions in litmus milk

TREATMENT OF SUSPENSION	PEPTONIZATION AFTER	
	8 hours	20 hours
	per cent	*per cent*
Unheated..............	50	100
Heated.....	0	0
Heated and held 1 week...........	0	0
Heated, toluene added, and held 1 week . ..	0	0
Heated, chloroform added, and held 1 week....	0	0
Unheated, chloroform added, and held 1 week	50	100

Reuhle (1923), basing his conclusions on data obtained with twice-washed bacterial spores, stated that the spores secreted enzymes even though they did not germinate.   Experiments were set up to determine whether such a mechanism was operative in the writers' own case.   It was found that suspensions of *Bacillus larvae* spores could be centrifuged and washed three times without being completely freed from proteolytic enzymes, as tested by inoculation into litmus milk.   Further washings, though, yielded suspensions showing no casein hydrolysis or gelatin liquefaction, even after prolonged incubation.

CONCLUSIONS

The above data indicate that the proteolytic enzymes found in honeybee larvae infected with *Bacillus larvae*, or in scales of

larvae dead from such infection, are not elaborated by the honey-bee larval organism. Larvae in the pre-sealed stage, at which time they are consuming pollen and honey, do contain enzymes which liquefy gelatin and peptonize milk, but in healthy larvae, after being sealed and in the prepupal stage, the enzymes can no longer be demonstrated. That these enzymes are released by the bacterial organism as it sporulates is evidenced by the fact that the amount of enzyme in diseased larvae is in direct proportion to the percentage of spores, and although in vegetative cultures of *B. larvae* no such proteolytic enzymes are found, they appear concomitantly with sporulation, increasing with further sporulation. Production of these enzymes by dormant spores could not be demonstrated.

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THE ACTIVITY OF CHEMOTHERAPEUTIC AGENTS

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Studies conducted *in vitro* have contributed considerable information regarding the quantitative aspects of the problem of the action of chemotherapeutic agents on microörganisms. McIntosh and Whitby (1939), Chandler and Janeway (1939), Bullowa, Osgood, Bukantz and Brownlee (1940) and others have pointed out that there appears to be a quantitative relationship between the effective bacteriostatic concentration of a chemotherapeutic agent and the number of bacteria. However, most of the methods employed in studying the *in vitro* action of drugs do not lend themselves to a precise evaluation of the results because of their inherent errors. For example, plate counts cannot be relied on to demonstrate small differences in bacterial populations. It will be shown subsequently that small differences in population numbers may be of importance in interpreting the effects of drugs on susceptible microörganisms during the first two to four hours of incubation.

One of the characteristics of a chemotherapeutic agent is its bacteriostatic or bactericidal action on susceptible organisms *in vitro*. Whether the action is bacteriostatic or is bactericidal appears to depend on the environmental conditions. Under favorable growth conditions the apparent effect is a slowing in the rate of increase, whereas under unfavorable conditions the effect is apparently bactericidal. It will be pointed out later that the data presented in this paper may be interpreted as indicating that the action of the drug is bactericidal even though the organisms are grown in a favorable media. It is the purpose of this report to present in detail a simple and rapid method for studying and demonstrating the *in vitro* activity of chemotherapeutic agents and interpreting the data thus obtained.

METHOD

After an extended series of experiments the following procedure was adopted for the routine testing of the *in vitro* activity of various chemotherapeutic agents:

Growth media. All microörganisms were grown in a beef-heart infusion broth containing 0.2 per cent glucose and 1.0 per cent peptone. Occasionally it was necessary to fortify this medium with 1.0 per cent horse serum in order to obtain optimal growth conditions.

Cultures used for inoculum. Only cultures in the logarithmic growth phase were used to inoculate the drug and control tubes; in most instances this was a three- or four-hour culture. Where possible, mouse-passed cultures were employed.

Preparation of tubes for testing. Eighteen tubes, each containing 10.0 ml. volumes of broth, were prepared for each test as follows: three tubes of each of the following concentrations of drug—50, 30, 20, 10, 5 and 0 mg. per 100 ml. Where the solubility of the drug precluded the preparation of the above concentrations a more restricted range of concentrations was run.

Size of inoculum. One each of the above series of tubes was inoculated with 50 million organisms, one with 25 million and the third with 12.5 million organisms. The volume of inoculum from the three- or four-hour culture tubes to give the above population density was determined by means of photronreflectometric turbidity readings (Libby, 1938). The turbidity readings in most instances were correlated with population numbers by means of slide and plate counts; however, this is not essential for the method given in this report.

It will be shown subsequently that the amount of inoculum used to study the *in vitro* activity of chemotherapeutic agents is controlled in many instances by the methods employed.

Determination of population numbers. The increase in population numbers for the drug and control tubes was obtained by taking photronreflectometric turbidity readings after one-, two-, three- and four-hour incubation periods. The total population numbers were expressed directly in turbidity readings. For comparative purposes the activity of various drugs is expressed

as a per cent of the control population at the four-hour incubation period.

All turbidity determinations were restricted to a narrow range of galvanometer readings to insure their linearity.   This was accomplished by making appropriate dilutions.

<div align="center">EXPERIMENTAL</div>

The effect of active drugs on the rate of increase in population numbers during the first four hours of incubation.   Figure 1 shows a
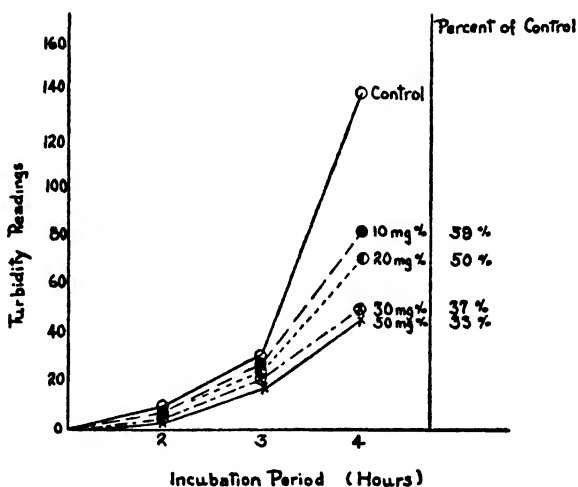
FIG. 1. GROWTH OF A TYPE I PNEUMOCOCCUS CULTURE IN BROTH WITH AND WITHOUT SULFATHIAZOLE IN VARYING CONCENTRATIONS
(Growth expressed in arbitrary turbidity units); (Inoculum 25 million organisms in 10 ml.)

typical series of data obtained by growing a Type II pneumococcus culture (inoculum 25 million organisms) in 10.0 ml. broth tubes containing 50, 30, 20, 10 and 0 mgm. per cent concentrations of sulfathiazole.   The population numbers are expressed directly in turbidity readings; a two-fold increase in the reading indicates that the population number was doubled.

The data given in this curve show that there was a rapid multiplication of the organisms in both the control and drug tubes during the four hours of incubation.   There is no indication of any lag period.   However, it is apparent that the population

numbers in the control tube exceed those in the drug tubes for the two-, three-, and four-hour incubation periods. Thus, for example, after four hours of incubation the population in the tube containing 50 mgm. per cent drug concentration was only 33 per cent of the number of organisms in the control tube. With decreasing drug concentrations the population numbers increase but are always less than the number of organisms in the control tube.

Figure 2 shows in more detail the relation between the concentration of drug and population numbers during the first four hours of incubation. The relationship is expressed by plotting the per cent of control population for the various drug concen-
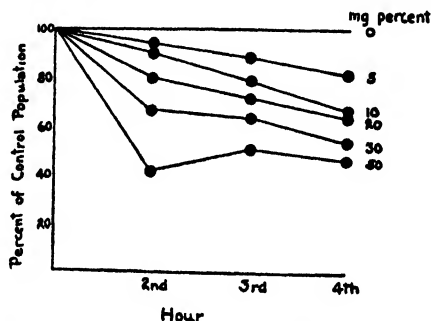
FIG. 2. PER CENT OF CONTROL POPULATION AFTER 2, 3, AND 4 HOURS INCUBATION
(Composite of 20 tests); (type II pneumococcus)

trations after two, three and four hours incubation. For example, the population numbers in the tube containing a 20 mgm. per cent concentration of drug was 80 per cent of the control population after two hours incubation, 73 per cent after three hours and 65 per cent after four hours incubation. Thus, with the passage of time and the increase in population numbers, the effect of the drug becomes progressively more marked. This relationship holds for all concentrations of drug except the 50 mgm. per cent concentration. In this instance the effectiveness of the drug was more pronounced during the first two hours.

The significance of the apparent change in the slope of the curves with increasing concentrations of drug is being investigated further.

The relation between the concentration of drug and the per cent of the control population. Table 1 and figure 3 present a typical series of data showing the effect of varying concentrations of drug

TABLE 1

*Effect of varying concentrations of drug on the per cent of the control population**

DRUG AND ORGANISM TESTED	PER CENT OF CONTROL POPULATION AT A DRUG CONCENTRATION OF:				
	50 mgm. per cent	30 mgm. per cent	20 mgm. per cent	10 mgm. per cent	5 mgm. per cent
Type II pneumococcus:					
Sulfanilamide....................	59	68	74	77	88
Sulfapyridine....................	52	57	59	64	69
Sulfathiazole....................	39	54	61	66	70
Streptococcus C493:					
Sulfapyridine........	38	43	49	47	55
Sulfathiazole..........	39	42	44	46	50
Sulfanilamide.	48	50	49	47	51
L–241................	16	30	41	46	51
S–11..........	25	26	37	43	47
Staph. aureus:					
Sulfathiazole.	38	41	44	50	57
L–241................	16	32	38	56	64
Flexner (6) dysentery:					
Sulfathiazole.......	15	25	30	35	40
Paratyphoid A (3):					
Sulfanilamide...... .. .	79	80	89	90	97
Sulfapyridine...	64	63	64	67	72
Sulfathiazole	26	38	46	52	58

* Data for the 4-hour incubation period.
L–241 = pp' diamino diphenyl sulfone.
S–11 = N⁴-sulfanilyl sulfanilamide.

on the per cent of the control population for a four-hour incubation period.

Table 1 shows that as the concentration of drug increases the effectiveness in retarding the increase of susceptible organisms becomes progressively greater.

If the concentration of drug in milligrams per cent and the

logarithm of the per cent of the control population are plotted, as shown in figure 3, a linear graph is obtained. This linear relation between the concentration of drug and the logarithm of the per cent of the control population indicates, that, under the experimental conditions employed in this study, there is a direct relationship between the concentration of drug and its effectiveness.
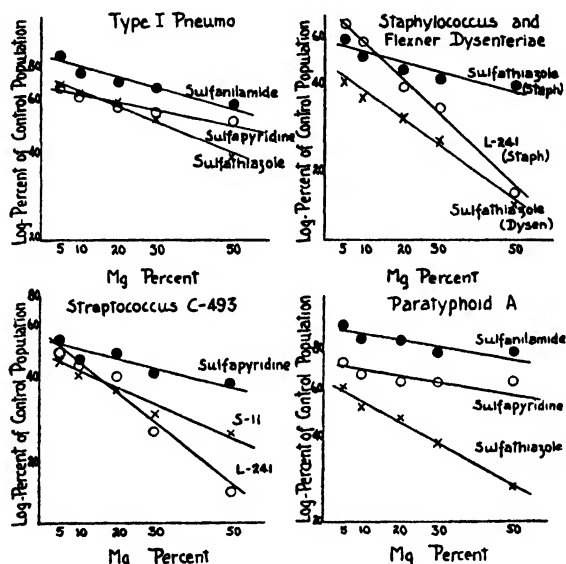
FIG. 3. RELATIONSHIP BETWEEN THE CONCENTRATION OF DRUG AND THE PER CENT OF THE CONTROL POPULATION
(Data from table 1)

The data in figure 3 also indicate that the mode of action of various sulphonamide drugs on various susceptible microörganisms is essentially the same.

The range of concentrations of drug in which the above proportionality exists is dependent on the degree of activity of the drug and the susceptibility of the organism used. Data showing the relationship between the degree of activity of drugs and the effectiveness of varying concentrations will be presented in a subsequent report.

The relation between the inoculum and the per cent of the control population. Table 2 shows that as the inoculum is decreased

from 50 to 25 to 12.5 million organisms the same concentration of drug exerts a progressively greater effect.

If the inoculum and the logarithm of the per cent of the control population are plotted, as shown in figure 4, a linear graph is obtained. This linear relationship between the size of the initial

TABLE 2

*Effect of varying the number of organisms inoculated on the per cent of the control population**

DRUG AND ORGANISM TESTED	PER CENT OF CONTROL POPULATION WITH AN INOCULUM† OF:		
	50 million per 10 ml.	25 million per 10 ml.	12.5 million per 10 ml.
Type II pneumococcus:			
Sulfanilamide.	87	72	69
Sulfapyridine.	61	51	44
Sulfathiazole.	56	52	45
S–11.	77	72	57
L–241.	54	44	38
Paratyphoid A:			
Sulfathiazole.	47	39	35
Sulfapyridine.	77	57	46
Streptococcus C493:			
Sulfanilamide	68	57	34
S–40	69	60	40
Flexner (6) dysentery:			
Sulfathiazole	33	27	26
Average.	63	53	43

* Data for the 4-hour incubation period.
† Per 10 ml. of broth.
S–11 = pp′ diamino diphenyl sulfone.
L–241 = N⁴-sulfanilyl sulfanilamide.

inoculum and the per cent of the control population indicates, that, under the experimental conditions employed in this study, there is an indirect relationship between the size of the inoculum and the effect of the drug.

The same limitations, as noted in the previous section, apply to the relationship between size of the initial inoculum and the effectiveness of the drug.

Comparison between concurrent plate and turbidity counts.
There is a fundamental difference between turbidity counts and
plate counts of a suspension of organisms.   The counts obtained
by means of turbidity readings give the total population, whereas,
plate counts give only the number of viable organisms in the total
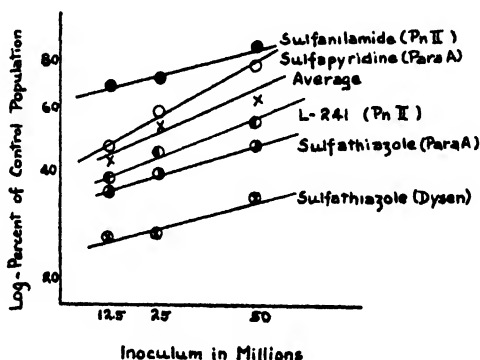
FIG. 4. RELATIONSHIP BETWEEN THE NUMBER OF ORGANISMS INOCULATED AND
THE PER CENT OF CONTROL POPULATION
(Data from table 2)
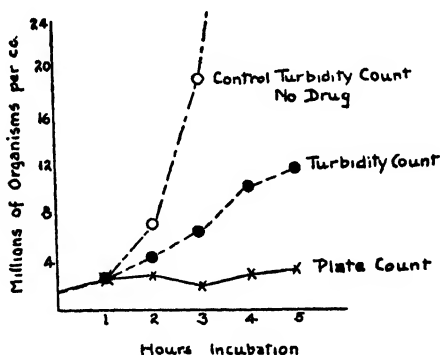
FIG. 5. COMPARISON BETWEEN TOTAL POPULATION AND VIABLE POPULATION IN
THE PRESENCE OF DRUG
(Sulfathioazole 50 mgm. per cent and Flexner dysentery)

population or, more strictly speaking, the number of organisms
capable of reproduction.   Therefore, if it can be shown that the
total population increases in the presence of an active chemo-
therapeutic agent at a greater rate than the number of viable
organisms, then a certain percentage of the total population must

be dying or rendered incapable of reproduction. Some preliminary work has been started to test the above premise.

Figure 5 indicates the type of data that have been obtained in preliminary experiments in which concurrent turbidity and plate counts have been made. As shown in this figure there was no significant increase in the number of viable organisms between two and five hours as indicated by the plate counts but during this same period the total population, as indicated by the turbidity readings, increased about three times.

DISCUSSION

In a number of important respects the data presented in this report are not in agreement with the generally accepted views of the activity of chemotherapeutic agents *in vitro*. It has been generally accepted that it is desirable, if not necessary, to use a small initial inoculum in order to demonstrate the bacteriostatic or bactericidal properties of chemotherapeutic agents *in vitro* (Long and Bliss, 1939) (Colebrook, Buttle and O'Meara, 1936). A comparatively large initial inoculum has been used in the method outlined in this report. It has also been generally accepted that there is a period of from 2 to 6 hours before the *in vitro* effect of a drug becomes apparent (Long and Bliss, 1939) (Lockwood, 1938) (Osgood, 1938) (Spring, Lowell and Finland, 1940) (Whitby, 1938). All of our *in vitro* studies, with the method outlined above, on the activity of chemotherapeutic agents have been made during the first four hours of incubation. In every instance where a drug has shown activity at the four-hour incubation period it has been possible to demonstrate activity at the two-hour incubation period. It was shown in figures 2 and 3 that the effect of a chemotherapeutic agent *in vitro* was indirectly related to the size of the inoculum and directly related to the concentration of the drug under the conditions of the experiment. Thus, one would expect, and does find reported in the literature, all degrees of activity from that of a retardation in the rate of increase to bactericidal activity, depending on the relative proportions of drug and organisms in any one test.

Whether the bacteriostatic effect or the bactericidal effect of

the drug is selected for study is determined partially by the method employed. Large inocula are used in our studies for two reasons; first it was found that only under these conditions was it possible to start and complete an *in vitro* drug test in one working day of eight hours and second, large inocula were necessary in order to obtain one-hour and two-hour turbidity readings on the Photronreflectometer (Libby, 1938). Before the method was adopted it was demonstrated that similar results could be obtained by using small inocula but in this case it was found that it was necessary to extend the time of observation to 12, 24 or more hours.

In some of the methods used to demonstrate the *in vitro* activity of chemotherapeutic agents a small inoculum is an essential component of the technic (Fleming, 1938) (Osgood, 1938). In many instances where plate counts are used to determine the activity of a drug, control and drug readings are taken only after incubation of the culture for twenty-four hours or more; during the first few hours the bacterial population increases in both the drug and control tubes (Lockwood, 1938) (Spring, Lowell and Finland, 1940). Thus, in this case the activity of the drug on relatively large numbers of organisms is actually being studied.

The differences in population densities between the drug and control tubes at the two-hour incubation period, as shown in figure 1, are small. With less active drugs or less susceptible organisms than those shown in this figure the differences are even less. In general the population densities in the drug tubes are anywhere from 2 to 20 per cent less than in the control tube at this period. However, on repeated tests these differences are constant. If plate counts were used to determine the activity of the drug at the two-hour period it would be difficult to establish any constant difference between the drug and control tubes since the accuracy of repeated plate counts in many instances is within this range of difference.

There is no indication in our data of the existence of a lag phase before the activity of the drug becomes apparent. This is probably due to the fact that small differences in population density

can be detected by Photronreflectometric determinations and the fact that by this method both viable and nonviable organisms are estimated.

The existence or nonexistence of a lag phase prior to the apparent action of a chemotherapeutic agent on a susceptible microörganism plays an important rôle in interpreting the mode of action of the drug. A number of theories have incorporated the assumption that a lag phase does exist (McIntosh and Whitby, 1939) (Locke, Main and Mellon, 1938) (Long and Bliss, 1939).

It has been generally agreed that the *in vitro* action of chemotherapeutic agents is to retard the growth of susceptible microorganisms. This action may become bactericidal, if sufficiently high concentrations of drug are used, or if the chemical or physical environment is rendered sufficiently adverse in itself to retard growth. If the action is retardation of growth, then to apply this concept to the data presented in this paper it must be assumed that, as the population increases, the retarding action becomes increasingly effective during the first four hours of incubation (see fig. 1).

McIntosh and Whitby (1939) and others have reported that the action of sulphonamide drugs on microörganisms cannot be a germicidal one because of the existence of a "lag phase" in contrast to the immediate action exhibited by true germicides. If, as pointed out above, one employs a technic that will distinguish small differences in population densities between the drug and control tubes during the first few hours of incubation it is possible to demonstrate that the action of the drug becomes apparent immediately. Thus it becomes reasonable to postulate that the action of sulphonamide drugs is a bactericidal one and that this effect takes place immediately on a small percentage of susceptible organisms. It is generally agreed that adverse environmental conditions enhance the apparent action of drugs. If this is true one would expect to find, with the increase in population density and the passage of time, that the effect of the drug would become more apparent. The data presented in this report support the above observations.

SUMMARY

A simple and rapid method for demonstrating the *in vitro* activity of chemotherapeutic agents on susceptible micro-organisms during the first four hours of incubation has been given in detail in this report. Essentially, the method involves a comparison of the population densities in drug and control tubes during a four-hour incubation period. The population densities were determined by means of photronreflectometric turbidity readings.

An analysis of the data obtained by means of the above technic showed that:

1. There was a rapid multiplication of the organisms in both the control and drug tubes during the four-hour incubation period. However, the population numbers in the control tube exceeded those in the drug tubes. The difference in population numbers between the drug and control tubes was small after two hours of incubation but became progressively greater with increased incubation time up to four hours.

2. There was no indication of a lag phase prior to the apparent action of the drugs on susceptible organisms.

3. The effect of the drugs on susceptible organisms was directly related to the concentration of the drug, within certain limits prescribed by the degree of activity of the drugs and the susceptibility of the organism used in the test.

4. The effect of the drugs on susceptible organisms was indirectly related to the size of the initial inoculum.

5. There appears to be a more rapid increase in the total population as indicated by photronreflectometric turbidity readings than in the number of viable organisms as indicated by plate counts when susceptible organisms are grown in the presence of highly active drugs.

The mode of action of chemotherapeutic agents as indicated by the data presented in this report is discussed.

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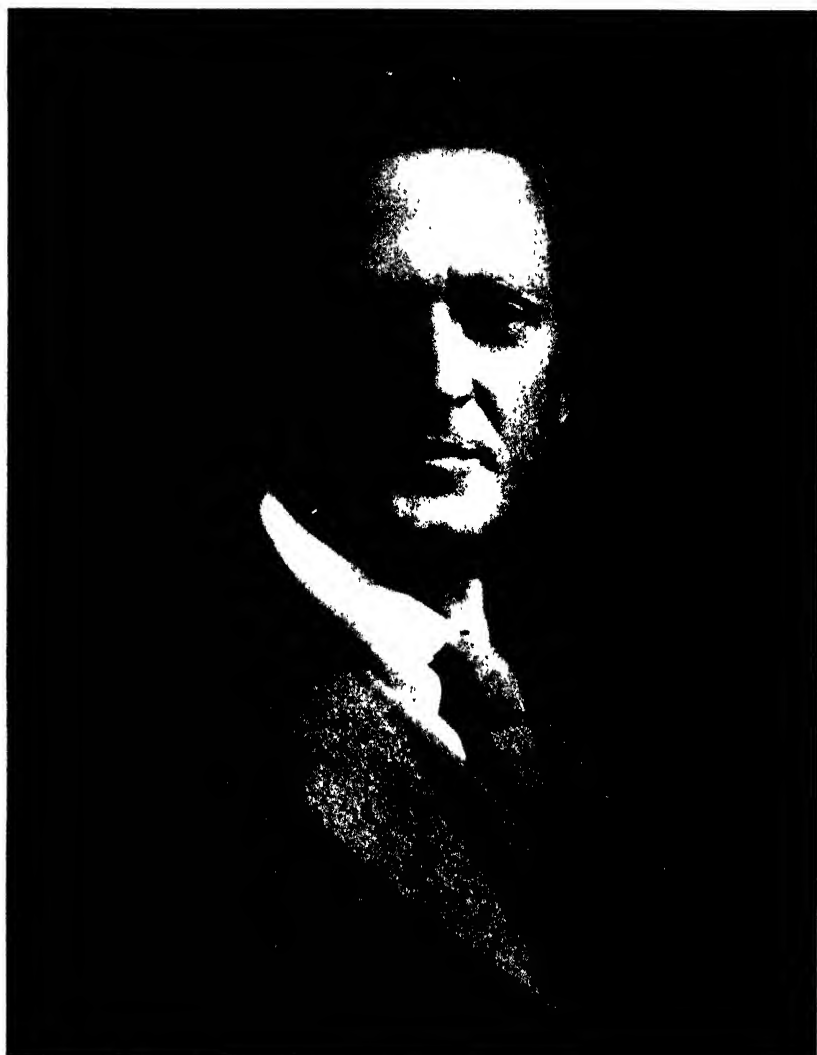
Hans Zinsser
1878–1940

𝕳𝖆𝖓𝖘 𝖅𝖎𝖓𝖘𝖘𝖊𝖗[1]
1878-1940

After a two years' illness, during which his courage made possible an almost normal continuation of his manifold undertakings, Hans Zinsser died on September fourth.   It was his wish that he might work, unharassed by the sympathy and concern of his friends, normally and without fear until the end.   To this purpose we few, who were his constant associates, were bound to silence, and so well did he maintain his stand that many in daily association with him had no suspicion of the facts.   The personal loss is not for us alone, but is shared by the thousands of physicians whom he has taught and stimulated during more than thirty years, and tens of thousands, medical and lay, who knew him indirectly through his various activities.

Born in New York, November 1878, he was educated at Columbia University (A.B. 1899, A.M. 1903), then at the College of Physicians and Surgeons of Columbia University (M.D. 1903). After completing his interneship, he served as bacteriologist in the Roosevelt Hospital 1905-06, and during the same period was Assistant Bacteriologist at the College of Physicians and Surgeons, where he was gradually promoted to an Instructorship (1907-1910).   In 1910 he went to Stanford University, California as Associate Professor of Bacteriology and served as Professor there in 1911-13.   He was recalled to Columbia as Professor of Bacteriology in 1913 where he served for ten years, leaving in 1923 to accept the chair of Bacteriology and Immunology at the Harvard Medical School, which he filled until his death.   He held honorary degrees from Columbia, Western Reserve, Lehigh, Yale and Harvard.   He was a member of the American Red Cross Sanitary Commission to Serbia in 1915, Major and Colonel

in the Medical Corps in 1917–18, Sanitary Commissioner in Russia for the League of Nations' Health Section in 1923 and Exchange Professor to France in 1935 and to China in 1938. He received decorations from France and from Serbia and was awarded the Distinguished Service Medal of the United States.

At sixty-two Zinsser's mind was young and vigorous, and the list of his accomplishments, already overwhelming, could have been expected to reach to even higher peaks. By instinct and early training a master of literary style, he became fascinated by the unexplored paths of science while still an undergraduate, and, turning to medicine, was absorbed in the problems of infectious disease. A student, and later an associate of Philip Hiss, he found immediately in research, teaching, and the preparation of the manuscript for the Hiss and Zinsser textbook of medical bacteriology the natural outlet for his talents and dynamic energy. Reaching into an ever widening field, he became a pioneer in the newly opening science of immunology, and within a short time had organized classes in this subject and produced his second book, "Infection and Resistance."

From those early years until his death his activity continued unabated. Along with this teaching at Stanford, Columbia and Harvard an ever-increasing range of subjects have been illuminated by his keen insight, investigative skill and intelligence—work so well known to bacteriologists as to make review in detail superfluous. His investigations of anaphylaxis and immune phenomena of various sorts led to the adoption of the simplifying "unitarian" theory of the nature of antibodies. Tireless attempts to cultivate the organism of syphilis established the situation as it rests today. His "residue antigen" work developed into the new and remarkable knowledge of "soluble specific substances," and his investigation of bacterial allergy went far toward bringing order out of that chaotic subject. He was a pioneer in the measurement of the relative sizes of the invisible viruses. His typhus researches, so rich in theoretical and practical results, were perhaps those from which he derived the greatest satisfaction, and already he had begun to select new objectives,

delving into the obscurities of the filterable viruses and planning an approach to a study of the dread disease, leprosy.

These achievements in teaching and research by no means exhausted his capacities. He became one of the foremost authorities on epidemiology and public health as related to infectious diseases. Military sanitation in 1917–18 with the Second Army, and field investigations of typhus fever in Serbia, Russia, Mexico and China claimed his attention. He gained richly in experience from each such expedition, but returned full measure in practical results.

Throughout, his love for literary expression, his happy mode of speech and the play of his active mind resulted in perpetual charm to many an audience and to myriads of unseen readers. In addition to his contributions in the scientific field, he wrote and spoke of many subjects,— religion and poetry, history and education. Every bacteriologist reads his "Rats, Lice and History," and his very recent autobiography "As I Remember Him." Many, too, have doubtless been moved by an occasional sonnet in the Atlantic Monthly signed merely "R. S." without realizing that its author was, like themselves, a bacteriologist.

These are the aspects of Hans Zinsser's career which are familiar to most bacteriologists, but they do not tell the whole story. On October 8th, 1940 a meeting in his memory was held at the Harvard Medical School when Dr. John F. Enders read the following paragraphs concerned with that more intimate knowledge derived from long personal association which we in the laboratory were fortunate to share. They describe something of this side of a remarkable character known to many but indirectly through his accomplishments.

"Various were the attributes of Hans Zinsser's luminous mind and spirit. In thinking of him to-day, some of you see him as a great teacher, others as a distinguished investigator, many as a perspicuous and sensitive commentator on science, letters, education, and on his own and others' lives. An increasing number recognize in him a poet of great emotional intensity. But a

comparatively small group of us—possibly the most fortunate of all—were with him in the laboratory. We were his friends, and with him shared the task. I am the inexpert spokesman for these privileged people.

"I want to speak mostly of him as head of the laboratory; to depict as best I can some of these qualities of mind and heart which enabled him to fashion that office into a remarkable instrument whereby not merely bacteriology and immunology were advanced, but the lives of those who came to him were broadened and enriched. It is a truism which cannot be too often repeated that no man can arouse the enthusiasm of others and lead them into the delights of his chosen paths unless he himself burns with the fires of devotion. In Hans Zinsser this flame burned with an extraordinary brilliance and, like a beacon, attracted others from all over the earth. For more than three decades, every day and frequently at night, he was in the laboratory until the very last. And he was here because he could not stay away. The problem of the moment absorbed him completely. It broke his sleep and dragged him willy-nilly to his experimental animals and his cultures. Its progress, as he has said, largely governed his mood, which was either 'himmelhoch jauchzend' or 'zum Tode betrübt,' depending on the success or failure of his experiments. He has himself told us how he looked upon his work: 'There is in this profession,' he says 'a fascination which holds the spirit with feelings that are not exaggerated by the word "passion"; indeed, like the happiest personal passions, it feeds on the intimate daily association of long years and grows—like love—with an increasing familiarity that never becomes complete knowledge. For what can be more happily exciting than to study a disease in all its natural manifestations?'

"But this is not the only, nor perhaps the most significant aspect of his attitude toward his profession, for many—according to their natures and capacities—are thus enslaved. His gallant and imaginative spirit saw in scientific research high adventure. The investigation of infectious disease became for him a field of battle. Always loving and even often seeking out a struggle where benevolent causes were at stake, this lifelong conflict with

the agents of syphilis, tuberculosis, typhus and the rest which he regarded perhaps only half-humorously as sentient malignities satisfied in large part his need for dangerous experience in the pursuit of generous ends. Those who surrounded him were set alight and newly energized by this flaming idealism.

"Also, we followed and loved him because of his complete lack of cant and his hatred of any trace of academic affectation and professional posing. This came, I am sure, not only from his own good sense—which he had in abundant measure—but also from his essential modesty and true humbleness of mind. More than most, he was aware of the broad lacunae in the knowledge of every man to-day, even in his own science. This made him eager to learn wherever he could. In a Cambridge undergraduate, a first year medical student, in a hostler, a fisherman or a Whitehead, he first saw a human being whom he possibly might help in some way, and then a source of information. With these purposes in mind, one can't for long preserve the professorial manner.

"But the extent of his general knowledge was sufficient to furnish forth respectably two or three professor's chairs. The world of letters only recently has become aware of his broad cultivation, but for us it was an old and delightful story. For some years we all lunched together in the laboratory. As we ate, the conversation—led by him—became animated. Literature, politics, history and science—all he discussed with spontaneity and without self-consciousness. Everything was illuminated by an apt allusion drawn from the most diverse sources, or by a witty tale. Voltaire seemed just around the corner, and Laurence Sterne upon the stair. Here, indeed, was a liberal education to be gained pleasantly, while one dined.

"Under such influences, the laboratory became much more than just a place to work and teach—it became a way of life. Coming here, I have seen younger men of considerable knowledge but little wisdom, still marked by lingering traces of adolescence, change almost suddenly into mature people aware of the infinite possibilities of existence. Often, he also brought about a rapid maturization of their latent capacities as independent investigators

through his deep wisdom in allowing those who showed some promise much freedom in working out their problems. Though always ready to help the neophyte with advice or a happy suggestion, he not only permitted him every opportunity for self dependence, but subtly urged him to develop his own powers to the top of his bent. Upon another he was seldom tempted to impose either his own ideas or his own will for he believed profoundly in the doctrine of intellectual and spiritual democracy, according to which it is the duty of the individual to follow to the end the beckonings of his own best genius.

"But more important still 'The Professor,' as we affectionately called him, became the friend of those who gathered about him. What he wrote of his colleague, Oscar Teague, is in double measure characteristic of himself: 'No one can know with exactitude the number of those who were helped by him according to their needs. His mind, his heart and his purse were open and accessible to those who needed them, and he is mourned-- with us--by many a laboratory boy and technician who has had his hand on his shoulder at a difficult time.' Constantly the quick sympathy of Hans Zinsser's friendly and affectionate nature expressed itself in acts of helpfulness.

"And, finally, shining out above all was his courage. Of his character this was the very essence. Always he sought the hazardous task, the dangerous place-- not without fear, for he confessed to being sometimes terribly afraid-- but because he knew that in its conquest was strength and peace.

"And so it would seem that all his life he was preparing himself for these last two years, when he came to know that the end could not be far away. We who were with him then will ever be the better for having seen him tranquilly continuing his researches, writing his books and teaching his students as if no term had been set. But the attainment of this inward calm was not as easy as he would have us believe, in what he has written. As the weeks and months passed, I know that it took the full measure of his brave soul to preserve it. Deeply we miss him, here in this place, and mourn the irreparable loss of his companionship and counsel. Yet the memory of the manner in which he faced

and met death compensates us in part. For, as a great poet has said of another who, suffering much, endured to the end:

> 'Nothing is here for tears, nothing to wail
> Or knock the breast; no weakness, no contempt,
> Dispraise or blame; nothing but well and fair,
> And what may quiet us in a death so noble.' "

Here, indeed, is the measure of a remarkable man who lived his life to the full,—student, philosopher, scientist, organizer and executive, poet, friend and companion. Seldom is it possible in a brief span of years to penetrate such diverse ways. Rarely can untimely death inflict a greater loss.

J. Howard Mueller.

CORYNEBACTERIUM DIPHTHERIAE

II. Observations and Dissociative Studies—the Potentialities of the Species[1]

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In spite of the fact that the diphtheria bacillus is one of the few species of microörganisms that have been extensively studied in the laboratory, it becomes necessary occasionally (especially during a period in bacteriology characterized by the birth of new theories relating to the nature and organization of bacteria), to repeat certain old experiments, as well as to perform new ones, for the purpose of collecting data which may either prove or disprove the applicability of the new theories to an individual species.

Practically every aspect of the diphtheria bacillus has, at one time or another, been the subject of dispute. The very fact that there are so many reported instances of variation in this species should immediately suggest that this organism would be especially suitable for studies in dissociative variation. It is true that there have been performed during recent times many studies in forced dissociation; but sometime during the studies, if they are to be broad and thorough, one must take cognizance of the reported cases of variation (Morton), and attempt some sort of correlation, rather than carrying the dissociative studies along certain narrow lines with the result, perhaps, of producing only additional and still unexplained instances of variation.

As a result of the recent studies on dissociative variation in many species and of the integrating concept of Hadley (1937), who has attempted to coördinate these observations, with the effect of producing a new picture of the nature of the bacterial individual and of the bacterial species, there has developed in modern bacteriology a tendency to depict bacterial species in a manner quite different from that heretofore demanded by the older monomorphic concept. The facts of pleomorphism and of the quite normal character of the chief dissociative culture phases, are now well established and fairly generally accepted, although the problem of the ontogenetic significance of these phases, as viewed by Hadley in his ontogenetic concept, can not receive a final answer at this time. It is with a tentative acceptance of the hypotheses indicated above that the present investigation of dissociative variation of the diphtheria species was undertaken and carried forward.

For some bacterial species there has been found a definite trend in the transformation process. In those species we are now able to correlate physical and chemical properties with such attributes as colony form. Instead of the existence of a chaotic state in the variation processes of these species, there can now be recognized a very orderly trend in the dissociation process. The thought therefore arises,—will the general scheme of dissociation of the diphtheria bacillus be the same as that for quite different species? Some workers have attempted to make such a general plan fit perfectly, with slight consideration for the special types of variations of *Corynebacterium diphtheriae*, already recorded in the literature (Morton).

The most fundamental new facts that one could contribute to our knowledge of the diphtheria bacillus would be those gleaned from a study of its life history. It is possible that a systematic study of its dissociative behavior would contribute much towards knowledge of this sort, rather than making the situation more complex. To date, no real comparative study has been made of the various culture phases of the diphtheria bacillus, in the light of the recent advances in bacteriology, especially along the lines of dissociative variation.

An extensive review of the literature has been made (Morton)

to ascertain the nature of the variations already reported for *C. diphtheriae*, since there was no previous attempt to interpret such variants from the standpoint of dissociation. As a result of this review, many instances have arisen in which, in the light of our present knowledge, it appears possible to correlate apparent discrepancies with colony form. These apparent correlations have been verified by studying both laboratory and freshly isolated strains. In some instances there has been employed a strain of *C. diphtheriae* derived from a single cell. There also have appeared variants which seemed to be independent of colony form, and this type of variation will also be considered. The plan has been to examine cultures of the diphtheria bacillus under conditions of "normal" cultivation, as well as subjected to various dissociative incitants; and to study any new forms which might result from such procedures.

Not always can a certain variation be produced at will. When a culture of the diphtheria bacillus is subjected to a particular dissociative incitant, perhaps a certain variant will occur; and in the course of study of this particular variant perhaps other variants will occur, and so on. After numerous investigations into various channels, it is well to stop and take inventory of the data in order to see what kind of composite picture the various parts make, with the aim of arriving at a better understanding of the nature of the diphtherial species—if not of the diphtheria "bacillus."

Anticipating somewhat the outcome of this investigation, it may be said that the results tend to confirm the concept of Hadley (1937) regarding the necessity of viewing in a new light the nature of bacterial species. The diphtherial species for example, is not typified exclusively by the "bacillus" of Klebs and Loeffler, but by a considerable diversity of morphological and cultural elements that, taken collectively, and perhaps (as Hadley has implied) in a certain sequence, make up the diphtherial *species-microphyte*.

MATERIALS

1. *Cultures.* Subcultures of the Park 8 strain were received from the Hygienic Laboratory, University of Michigan; Parke,

Davis and Company, Detroit, Michigan; Sharp and Dohme Laboratories, Glenolden, Pennsylvania and the Western Pennsylvania Hospital, Pittsburgh, Pennsylvania. A single-cell strain was derived from the Sharp and Dohme subculture of the Park 8 strain by the method described by Kahn (1929). Clinical strains were isolated from field cultures which had been submitted to the Department of Health, Detroit, Municipal Hospital, Philadelphia, and the Pennsylvania State Laboratory, Philadelphia. A few strains were also isolated from healthy "carriers." *Gravis* and *mitis* cultures were received from the National Drug Company, Philadelphia, and the New York City Department of Health. Some of the *gravis* and *mitis* cultures received as such were originally obtained from England.

Some of the clinical strains satisfied the criteria for the smooth colony type. As these studies were begun before the publication of Anderson, *et al.*, it is not known how those strains collected early in the studies appeared on potassium tellurite chocolate agar, but those strains collected more recently, when classified as smooth have appeared as *mitis* strains on the special medium. Those strains received as *mitis* strains either showed S colonies or a mixture of colony types in which S colonies could be found. All the Park 8 strains were intermediate between smooth and rough. One of the strains was rougher in appearance than the other three but it was not a typical R phase and so had to be classified as an intermediate. Those strains received as *gravis* strains showed colonies typical for such strains or a mixture of colony types in which *gravis*-type colonies could be found. The colonies appeared similar to the SR type colony. No strain was ever isolated from clinical sources which was as rough in appearance as R strains which were produced by forced dissociation.

From year to year some of the strains have shown variations in their colony form, exhibiting a mixture of colony types when plated. The stock cultures were maintained on blood agar slants, and in recent times have been overlayered with sterile paraffin oil (Morton and Pulaski, 1937) and transferred at intervals of six months to one year.

2. *Media.* Media prepared from a beef infusion base were

employed. In the beginning Bacto-peptone or Bacto-proteose peptone was employed but these have been discontinued in favor of Bacto-neopeptone or Parke, Davis & Co. bacteriologic peptone.

Infusion agar was prepared by adding two per cent agar to infusion broth.

Glycerol broth and glycerol agar were prepared by adding 5 per cent by volume of c. p. glycerol to filtered infusion broth or infusion agar.

Blood agar was prepared by adding 5 per cent by volume of sterile, defibrinated, normal rabbit or horse blood, aseptically, to infusion agar which had been melted previously and cooled to 45°C.

Tellurite chocolate agar was prepared as described in 1931 by Anderson, Happold, McLeod and Thomson or Bacto-tellurite blood solution and Bacto-dextrose proteose no. 3 agar was used.

Loeffler's medium was employed if sterilization was carried out in the autoclave. Intermittent sterilization in the inspissator was not trusted unless the medium was prepared by mixing the sterile ingredients under aseptic conditions and the inspissator used for coagulating the medium and not for sterilization. Hinkleman (1923) and Dupray (1924) have reported methods for sterilizing Loeffler's medium in the autoclave, but the methods were not found as satisfactory as that of maintaining constant pressure within the autoclave by means of compressed air during the heating and cooling of the autoclave.

Hiss serum water was prepared by adding six grams of Bacto-peptone or Parke, Davis and Company peptone to 900 ml. of tap water; the reaction was adjusted to pH 7.4 and the solution boiled vigorously for a few minutes. This procedure was done routinely, as certain batches of peptone coagulate serum upon mixing. The boiled peptone solution was restored to original volume and 300 ml. of beef or horse serum were added. Twenty-two milliliters of brom-cresol purple stock solution, 0.04 per cent, were added for the indicator, the medium filtered through filter paper and dispensed into sterile test tubes in 5 ml. amounts. Sterilization was carried out in the autoclave at about 105°C.

for 15 minutes and the medium incubated 48 hours to check sterility. This method of sterilization not only rendered the medium sterile but also destroyed any enzymes likely to be present in the serum which might hydrolyze certain carbohydrates and thus tend to false interpretations of the reactions of the cultures under test, as Goldsworthy, Still and Dumaresq (1938) have recently described. Sterile solutions of the various carbohydrates employed in the fermentation tests were prepared by dissolving either 5 or 10 grams (depending on the solubility of the carbohydrate in water) of the pure carbohydrate in 100 ml. of distilled water and filtering through a sterile Berkefeld "N" or sintered glass filter (Morton and Czarnetzky, 1937). The sterile filtrates were then dispensed into the tubes of sterile Hiss serum water in the amounts of 0.5 ml., if the concentration of the carbohydrate was 10 per cent, or 1 ml., if the concentration was 0.5 per cent. This gave a final concentration of the carbohydrate in the Hiss serum water of approximately one per cent. Extract broth has been found to be a better basic medium in the fermentation test if the strain is not too fastidious.

Before using, media which had been stored in the cold room or refrigerator, were warmed to room temperature.

<div align="center">EXPERIMENTAL</div>

1. *Influence of the reaction of the medium on the growth of the diphtheria bacillus.* Realizing that certain ranges in pH of the culture media are effective in bringing about, or stabilizing, certain colony types, one of the first things determined was the range in pH permitting growth of the diphtheria bacillus. The experiment was carried out by seeding one standard 4 mm. loopful of a 24-hour glycerol broth culture of the Park 8 strain, (Sr phase) into each of a series of tubes of glycerol broth of varying pH. The pH of the glycerol broth was carefully determined for each lot by means of the Leeds and Northrup quinhydrone pH indicator. The inoculated tubes of glycerol broth were incubated at 37°C. The results of the experiment are listed in table 1.

Practically the same results were obtained with a Park 8 strain from another source.

These results are comparable with those of Bunker (1919), who found that the diphtheria bacillus grew in a range of pH 5.7 to 8.7, and Dernby and David (1921), who found the range to be between pH 6.0 and 8.3 with the optimum between pH 7.2 and 7.8.

As a check on the macroscopic observations, glycerol agar plates were inoculated with three drops from the pH 5.6, 5.7, 6.2 and 8.5 tubes, respectively. The plate inoculated from the pH 8.5 tube showed no growth, whatsoever. The plate inoculated from the pH 6.2 tube showed numerous Sr colonies, which were typical of the strain when it was used as the inoculum for the

TABLE 1

Range in pH at which growth of C. diphtheriae, Park 8 strain took place

INCUBA-TION	pH 5.6	pH 5.7	pH 6.2	pH 6.6	pH 7.3	pH 7.7	pH 8.1	pH 8.5	pH 8.8
hours									
16	—	—	—	FC	C	—	—	—	—
24	—	—	—	FC	C	—	—	—	—
48	—	—	S, P	S, P	C, P	C, P	C, P	—	—
65	—	—	S, P	S, P	S, P	S, P	S, P	—	—
115	—	P*	S, P	S, P	S, P	S FC P	S, C P*	—	—

—, no visible macroscopic growth; FC, faint clouding; C, clouding; S, sediment; P, pellicle; * very small pellicle.

series of broth tubes. The plate inoculated from the pH 5.7 tube showed numerous very small colonies and only three large, typical colonies. The pH 5.6 tube showed only numerous very small colonies. Thus, small colony forms were encountered in the very first experiment in these studies. These particular small colonies will be described in detail in a later section.

2. *Serial transfer in alkaline glycerol broth.* It is generally known among bacteriologists interested in dissociation, that an alkaline medium usually favors the production of the rough culture phase. Therefore, continual cultivation of a strain in a favorable medium of the highest pH permitting growth is one of the commonest methods of producing transformation of a

strain to the rough phase. From the information obtained from the experiment in the immediately preceding section, serial transfers at 48-hour intervals were made of the Park 8 strain in tubes of glycerol infusion broth, pH 8.1. The serial transfers were carried on for 11 culture generations without evidence of change in the manner of growth in the broth or on glycerol agar plates. As the strain became adapted to the alkaline environment, more alkali was added to the tubes of glycerol broth in the form of sterile N/10 NaOH solution. By the twentieth culture generation, the strain was growing in the pH 8.1 glycerol infusion broth to which had been added 10 drops of sterile N/10 NaOH solution. Growth from this highly alkaline tube showed only the typical colonies which were present at the beginning of the series. The growth in the last tube in the series was allowed to evaporate to a resinous mass, over a period of 42 days, resuspended in sterile distilled water to the original volume of the broth and filtered through a sterile Berkefeld "N" filter under 34 cm. of negative pressure. At no time during the three months which elapsed did this filtrate give rise to growth on glycerol agar plates when treated by the Hauduroy (1927), technic, although positive results were obtained with other filtrates as will be described later.

3. *Serial transfer in acid glycerol broth.* Since serial transfer of the Park 8 strain in alkaline broth resulted in no change in the culture towards the R type, it was believed that serial transfer of the strain in acid glycerol broth might result in a purer S type culture. Serial transfer at 48-hour intervals of the Park 8 strain in glycerol broth of pH 6.2 for 23 culture generations resulted in no colonies which were smoother in appearance than those produced by the strain under ordinary conditions. Small colonies were encountered, however, which will be described in a later section.

4. *Heating at 39.5°C.* Roux and Yersin (1890) reported depriving a virulent diphtheria culture of its virulence by heating it at 39.5°C. for 25 days. Hewlett and Knight (1897) observed that heating at 45°C. for 24 hours killed all of the bacilli. Heating at 45°C. for 17 hours killed nearly all of the organisms; those that did live were completely changed "appearing like pseudo-

diphtheria bacilli." The organisms could be changed back to typical diphtheria bacilli by subculturing on serum at 37°C. for several generations.

Since it was found impossible to grow the Park 8 strain serially at a temperature of 43°C., the temperature of the incubator was lowered to 40°C. Throughout the experiment the temperature varied between 39.5 and 40°C. The strain was carried through 27 serial culture generations in glycerol infusion broth, covering a period of 32 days. The cultures and media were kept in the incubator except for about two minutes when the actual transfers to fresh media were made. There was no change in the manner of growth of the organisms in broth or on glycerol agar throughout the series. One and eight-tenths milliliters of the 24-hour culture from the twenty-seventh tube in the series was inoculated into a 280-gram guinea pig. As a control 2 ml. of a 24-hour broth culture of the parent strain grown at 37°C. was injected into a second guinea pig. Both animals were dead at the end of 40 hours. Typical diphtheritic post-mortem lesions were found.

Two milliliters of the culture from the first tube in the series, which had been kept at about 40°C. for the entire 32 days, proved to be non-pathogenic for a 315 gram guinea pig. This culture also failed to grow upon sub-culturing, which might explain the results obtained by Roux and Yersin.

5. *Effect of aging.* In a series of 25 broth cultures of the Park 8 strain contained in test tubes which were sealed as ampoules and allowed to age for 140 days at room temperature and in the dark, two of the ampoules (096 and 097) gave a smooth type of colony in addition to the typical SR colony of the Park 8 strain when the contents of the ampoules were streaked onto glycerol agar plates. The new, smooth colonies were round, convex, moist, smooth and shiny, with even margins. These smooth colonies were transferred to blood agar slants and grown in pure culture. They gave fermentation reactions typical for *C. diphtheriae*, were agglutinated by agglutinating serum prepared against the parent strain, but the cultures were not, at first, pathogenic for 250 gram guinea pigs. The strains were carried in stock as 096 and 097, respectively.

Numerous cultures of the diphtheria bacillus on solid media,

as they aged for weeks and months, were observed to show rough outgrowths from the margins of the intermediate colonies. No special attempt was made to isolate a rough strain by selectively culturing the rough-appearing secondary growth.

A series of 12 broth cultures of the Park 8 strain, Sr phase, were allowed to age at room temperature and in the dark. From time to time sterile distilled water was added to each tube to restore that lost by evaporation. Periodically, infusion agar plates were inoculated from the tubes. Only one of the 12 cultures eventually yielded rough variants. The same tube also produced the extremely small colony type.

6. *Growth of the Park 8 strain in the presence of antitoxin.* Several workers have reported that the growth of a toxigenic strain of the diphtheria bacillus in the presence of antitoxin has resulted in the strain becoming non-toxic. To determine whether or not such a change would be accompanied by any other change in the organisms, the Park 8 strain was grown serially in the presence of antitoxin. The diphtheria antitoxic horse serum was obtained from a diphtheria antitoxin-producing horse which was showing a potency of 900 units per milliliter of serum. The first 7 tubes in the series contained 10 per cent antitoxic serum in glycerol infusion broth. The next 5 tubes contained 20 per cent serum; the next two tubes, 50 per cent serum, the next tube, 75 per cent serum and the last three tubes in the series contained the undiluted antitoxic serum. In all, the strain passed through 18 culture generations in the presence of antitoxic antibodies. At no time did the strain show any variation in its colony form. A tube of plain glycerol broth was inoculated from the last tube in the series and incubated for 48 hours, the purpose of this tube being to eliminate the possibility of any antitoxin being transferred along with the inoculum when the strain was tested for toxin production. A flask of Bacto-proteose peptone infusion broth was inoculated from the 48-hour glycerol broth culture and, for a control, a second and similar flask of proteose peptone infusion broth was inoculated from a glycerol broth culture of the parent Park 8 strain maintained on Loeffler's medium. Both flasks were incubated at 37°C. for eight days and both showed the

characteristic pellicle and sediment with no clouding of the broth. Both cultures were filtered through a sterile Chamberland L3 filter under a negative pressure of not more than 5 cm. mercury. To a portion of each filtrate was added tricresol to the amount of 0.3 per cent and the toxins placed in the cold room. After aging in the cold room for two weeks, the toxins were injected in varying amounts into guinea pigs with the result that both toxins were of practically the same potency, having an M.L.D. of 0.02 ml.

From this experiment it was concluded (1) that the toxigenic Park 8 strain of *C. diphtheriae* does not necessarily lose its toxigenicity when grown in the presence of antitoxin; (2) that the Sr colony type of the Park 8 strain is not markedly changed by the growth of the organisms in the presence of antitoxic antibodies; and (3) that these observations corroborate the findings of Schick and Ersettig and of Yü, but are contrary to the findings of Bernhardt (1914), Becker, (1927) and Jungeblut (1927).

7. Growth of the Park 8 strain in the presence of anti-bacterial serum. The Park 8 strain was grown for 21 culture generations in the presence of its homologous immune serum without any change, whatsoever, occurring in the manner of growth of the strain in liquid or on solid media. Since the serum did not show complete agglutination in dilutions of 1:100, another attempt was made by growing the strain for 16 culture generations in the presence of a serum showing complete agglutination in a dilution of 1:1280. No change in the character of the strain was produced. Another serum showing partial agglutination in a dilution of 1:4,000 did not change the character of the strain after growing the organisms in the undiluted serum for eight culture generations. Finally a serum showing definite agglutination in a dilution of 1:10,240 was used against a Park 8 strain from another source. The organisms were grown for 24 culture generations in broth containing 50 per cent immune serum with no change in the appearances of the strain.

8. The effect of growing the diphtheria bacillus in the presence of lithium chloride. a. Park 8 strain. Growth of the Park 8 strain in glycerol infusion broth containing 0.5 per cent or more

of lithium chloride resulted in the production of small colonies, which will be described in a later section. No rough colonies were encountered with this strain.

b. A freshly isolated strain.[2] Since the Park 8 strain had failed to produce the rough colony form by the usual methods employed to produce the rough forms of other organisms, it was thought that perhaps a strain of the diphtheria bacillus freshly isolated from a patient might be more easily dissociated.

For the experiment a strain isolated from a field culture, definitely identified as a true diphtheria bacillus and carried in stock as *C. diphtheriae*, II, was employed. On plain agar it gave the smooth type of colony. This strain was inoculated into tubes of infusion broth containing one per cent LiCl plus one drop of sterile 50 per cent LiCl solution. Upon inoculating a one per cent LiCl agar plate from the second tube in this series, rough-appearing colonies were observed. The roughest colony was spread over the surface of another LiCl agar plate and the roughest colony on this plate transferred to a third LiCl agar plate, on which the colonies were uniformly rough. One of the roughest colonies was then transferred to a tube of 1 per cent LiCl broth. The growth from this tube was plated onto infusion agar and the roughest colony transferred from this plate to another tube of 1 per cent LiCl broth. This process was repeated for three more culture generations. It was thought that the selection of the roughest colony on each plate to serve as the inoculum for the broth tubes would hasten the change of the colony form of the strain to the extreme rough type. The roughest colony on the last plate in the series was again picked from the plate to another plate of LiCl agar and this showed all rough colonies. Again one of the roughest colonies was picked to another LiCl agar plate and this also showed all rough colonies. This demonstrated that the strain was producing only rough colonies. A rough colony was picked off the plate to a tube of one per cent LiCl broth plus two drops of sterile 50 per cent LiCl solution.

The organisms grew as a granular sediment on the bottom of the tube. The strain was carried serially through 5 more tubes of 1 per cent LiCl broth each containing two drops of sterile 50 per cent LiCl solution. (The strain would not grow in the presence of more LiCl.) Upon plating out the growth in this tube, both smooth and rough colonies appeared. A rough colony was picked from this plate and carried serially through 8 more tubes of 1 per cent LiCl broth. The strain now showed upon plating, only extremely rough colonies. A glycerol agar plate and an infusion agar plate were inoculated from the same LiCl broth tube in this series. The infusion agar plate showed very rough colonies, whereas the colonies on the glycerol agar plate were not nearly as rough in their appearance. Similar parallel platings on glycerol agar and infusion agar throughout the experiment confirmed this observation and the observations of Zupnik (1897) and Schick and Ersettig (1903) that glycerol agar does not satisfactorily differentiate the rough and smooth colony types of *C. diphtheriae*. A guinea pig inoculated with all of the 24-hour growth of the rough culture from a blood agar slant died in 24 hours.

Believing that the culture was again fairly well established in the rough colony form, it was inoculated into plain infusion broth. Upon plating, this culture yielded only very rough colonies, one of which was fished to a tube of infusion broth. Upon plating this culture, only very rough colonies were obtained. The strain was passed through 4 more culture generations in infusion media and then inoculated to a blood agar slant and kept over the summer. In the fall this three months' old culture was carried through a series of four 1 per cent LiCl broth tubes. The first tubes in the series showed only rough colonies but the fourth tube showed 40 large smooth colonies scattered over the plate, which also contained small, extremely rough colonies, too numerous to count. A rough colony was then transferred to a tube of 1 per cent LiCl broth, the culture plated out and the roughest colony on the plate reseeded into a 1 per cent LiCl broth tube. This process was carried on for 9 culture genera-

tions. All of the cultures showed only the very rough, small colonies. A rough colony was transferred to a plain agar slant. This slant, after incubation, showed two raised, round, creamy, smooth colonies among the countless flat, irregular, bluish, rough colonies. A smooth colony was selected and grown in a pure culture, which was the third instance in which the colony type reverted to the smooth type after the strain had shown only rough colonies for several generations. The typical rough colonies were transferred to infusion agar slants and when plated on infusion agar plates yielded only extremely rough colonies. A culture of the colony pictured in plate I was propagated as the II R strain.

Many of the plates made from LiCl broth cultures showed numerous extremely small colonies, 0.2 mm. or less in diameter. Nothing was done with these, as they had been studied on other occasions and we were seeking the extreme rough colony phase.

In summary, it may be stated that the smooth, intermediate and rough colony types have been observed or produced in the following instances.

The smooth (S) colony type. The pure S has not been observed to be as common as the intermediate colony types in which the S characteristics predominate. The S colony type has been observed in field cultures, has been produced from the intermediate colony type by allowing broth cultures of the latter to age or by growing in media containing LiCl, and has been observed to arise spontaneously in cultures of the extreme R phase and by growing the latter in the presence of LiCl.

The intermediate colony type. Representatives of this group of intermediate colony forms were usually found to be present in strains of the diphtheria bacillus maintained on artificial culture media for a long time; it predominated in strains isolated from patients; it occasionally developed spontaneously from the rough colony type and also by culturing the rough type in the presence of LiCl. It might appear a bit unusual for one of the intermediate colony types to arise from the extreme R phase but this has been observed repeatedly, especially in stock cul-

tures of the R phase. Unless cultures in the R phase are maintained on solid media or occasionally grown in the presence of LiCl, or preserved under paraffin oil, or in the desiccated state, the very flat, bluish, irregular, granular colonies become thicker, more opaque, less irregular and show a central papilla.

The extreme rough (R) colony type. The extreme rough colony type was produced from the Sr type by aging broth cultures of the latter or by growing in the presence of LiCl.

APPEARANCE OF THE S, INTERMEDIATE AND R COLONY TYPES

Infusion agar. The S type colony is round and convex, with even margin and smooth surface. It is opaque when viewed by transmitted light, glistening and somewhat moist in appearance when viewed by reflected light. The growth is rather conservative on solid media as compared with the amount of growth produced by the intermediate type colonies. The colonies are usually about 1–3 mm. in diameter (plate I).

The intermediate type colonies comprise types intermediate between the pure smooth and rough phases, and have been designated by the symbols Sr, SR, and sR. In the transformation of S to R, the colony type slightly removed from the S phase has been referred to as the Sr type. The individual colonies are very similar to the S type colonies except that the surface is less convex, slightly drier, sometimes slightly pitted, and the margins may be slightly uneven and show a tendency to spread as the colonies age. The SR type colonies are distinctly larger than the S or R types, usually 3 5 mm. in diameter. By transmitted light the SR type colonies are opaque and when viewed by reflected light the surface is granular, not glistening, and drier than in the case of the S type colony. The surface is raised but not convex as in the case of the S type colony. Sometimes the surface is nearly level, at other times the central portion of the colony is raised and at other times there may be a central cone surrounded by a concentric depression and elevation. The margins usually show indentations. The growth is more luxuriant than with the other colony types and usually

shows a tendency to spread upon aging. The sR type colonies are thinner, the surface flatter and more uneven, the margins much more irregular and the colonies show a tendency to spread. The colonies very often show an opaque center which tapers off to a translucent margin, when viewed by transmitted light.

The extreme R type colony is smaller than the intermediate types and is usually slightly smaller than the S type, being about 1–3 mm. in diameter. The colony is flat and the margin very irregular. By transmitted light the colony is translucent; by reflected light the surface is pitted, very uneven and reflects little light. It is so flat and uneven that there was often insufficient contrast between it and the surface of the agar to permit photographing by reflected light.

Great difficulties may be encountered when describing colony forms if strict attention is not paid to the composition of the media. When comparisons were made, the various cultures were streaked upon plates poured from the same lot of media, prepared, incubated and examined under the same conditions and at the same time.

Colonies of the diphtheria bacillus, more than those of any other species of microorganism studied, varied greatly with the brand of peptone employed in the medium. Certain peptones, such as Bacto-neopeptone, Bacto-peptone, Bacto-proteose peptone, Chaissang's peptone, Medo-peptone, and Parke, Davis and Company's bacteriologic peptone, tend to produce moist colonies, some more so than others. Bacto-protone, Bacto-tryptone, Baker's peptone, Fairchild's peptone, Merck's peptone, Stearn's peptone and Witte's peptone yielded colonies very different from the ones obtained with the above mentioned peptones—some colonies so different that they would not have been judged diphtheria colonies by macroscopic examination had one not known the source of the culture. Some microorganisms grew nearly as well on plain extract agar without peptone as on the extract agar containing certain brands of peptone.

We have attempted to keep the composition of the culture media as simple as possible and still obtain good growth of the

diphtheria bacilli.   Infusion agar containing Bacto-peptone or
Parke, Davis and Company's peptone was used when it was
necessary to have a clear medium for viewing the colonies by
transmitted light.   Infusion agar containing 5–10 per cent fresh,
defibrinated rabbit blood was used for maintaining the cultures
in stock and whenever a medium richer than ordinary infusion
agar was required.   Colony types can be distinguished as well
on blood agar as on infusion agar.   Indeed, Wilson and Golds-
worthy have recently called attention to the fact that the *mitis*,
gravis and intermediate colony types of the English workers
can be distinguished very readily on infusion agar containing
Parke, Davis and Company's peptone and 5 per cent guinea
pig or rabbit blood.   There is no question as to the colony types
when one views their excellent photographs of the colonies on
blood agar.   The kind of blood, however, is very important in
differentiation of colony type, as Knox (1937) has shown.   Dud-
ley (1934) stated that trypsin serum agar gave a clearer differ-
entiation of colony types than did the potassium tellurite choco-
late agar.   Glycerol agar has been used a great deal but is not
favorable for the differentiation of colony form.

Potassium tellurite chocolate agar.   When grown on this special
medium the S type colonies appeared very much the same as
when grown on plain infusion agar except that the color of the
colonies was black.   The appearance of the S and *mitis* colonies
were the same.   Another striking similarity between the S and
mitis cultures was that both types were inhibited by the potas-
sium tellurite.   Many strains failed to grow when transferred
to this medium; other strains grew poorly.

The intermediate type colonies were very similar in appearance
to those grown on plain agar except for the color of the colony.
As the colony form approached the R phase, the color of the col-
onies became grayish.   The grayish appearance of the SR col-
onies was perhaps accounted for by the fact that the surface of
the colonies was pitted, drier and more uneven than in the case
of the S type colonies which were smooth and moist.   The SR
colonies and *gravis* colonies had the same general appearance

on the tellurite medium.   Another point in which the two types of colonies were similar was that they were not inhibited by the potassium tellurite, both growing luxuriantly.

The R type colonies were grayish, the color becoming more intense, sometimes, as the colonies aged.

TEXTURE OF SMOOTH, INTERMEDIATE AND ROUGH TYPE COLONIES

When a platinum needle was drawn through the S type colony, the colony, being moist, flowed over the needle, but the track of the needle did not disappear completely as when the needle is drawn, for example, through a drop of water.   If the needle was drawn through an SR type colony, the colony appeared drier because it did not flow over the needle.   Portions of the colony adhering to the needle did so as masses and did not appear to lose their shape or wet the needle.   The needle left a distinct groove, or furrow, which did not tend to close.   In the case of the R type, the colony was very dry and brittle.   When the needle was drawn through an R colony, a section of the colony often broke off from the colony and moved along in front of the needle.   Portions of the colony were picked up by the needle as particles which were hard to break up into finer particles.

APPEARANCE OF THE GROWTH IN LIQUID MEDIA

In these studies it has been observed that the S type colony produces a uniform turbidity in liquid media.

The intermediate type colonies, if near the S phase, Sr, produce both turbidity and sediment which is finely granular in contrast to the even turbidity produced by the S type.   As the intermediate stage approaches the R phase the granules become coarser and give rise earlier to a sediment, very little turbidity being produced.   The SR type produces an initial clouding of the broth which gives rise to a pellicle and sediment.

The extreme R type colony grows only as a very coarse, granular sediment.

Very similar to the appearance in broth was the appearance of the organisms of the various colony types when suspended in physiological salt solution.   The S type produced uniform,

stable suspensions, the SR and R types granular, unstable suspensions.

PRODUCTION AND DESCRIPTION OF THE D (DWARF) COLONY TYPE

Fourteen test tubes and four 125 ml. Erlenmeyer flasks containing infusion broth, pH 7.2, were inoculated with a Park 8 strain, whose colonies were of the intermediate type, and after incubating 48 hours to insure good growth, the cultures were placed at room temperature and in the dark. Naturally the cultures dried out as they aged so that sterile distilled water had to be added aseptically to restore the cultures to their original volumes before they were subcultured. The cultures in the tubes showed no evidence of growth in subcultures made after a period of 186 to 224 days. The flasks, after aging 227 days, were tested for viable organisms on January 11, 1934. Three of the flasks showed no growth in the subcultures. The fourth flask yielded about 500 colonies on the infusion agar plate. Some of the colonies were as large as 3 mm. in diameter, while about 25 per cent of the colonies varied from 0.13 to 0.2 mm. in diameter. The large and small colonies, when subcultured to blood infusion agar, maintained their relative sizes and both were smooth in appearance. The strains have been maintained on blood infusion agar slants and designated as O3–64 large and O3–64 small, respectively. The O3–64 large colony strain frequently has shown a mixture of large and small colony types upon plates which were made from old cultures. However, the organisms in the large colony type grow much more rapidly than do the organisms in the small colony type, so, if an old culture of the large colony type strain which might be showing a mixture of the two colony types is subcultured a few times at fairly short intervals, the resulting culture shows only the large colony type. The O3–64 small colony type strain has been found to be very stable. It has been maintained on blood infusion agar for over 6 years and the colonies are no larger than when first isolated in January, 1934. It is this dwarf colony type O3–64, derived from Park 8 strain which has been used in the experiments which follow for comparison with organisms from other colony types.

Both the O3–64 large and the O3–64 small colony strains fermented glucose and dextrin but not sucrose. The small colony strain, however, required a longer time to bring about the reaction. Both colony types were pathogenic for guinea pigs. The large colony type killed each of two guinea pigs in less than 24 hours. The small colony type, when injected into two guinea pigs produced death in slightly less than 70 hours and 10 days, respectively. Both strains were agglutinated to the same titer by immune rabbit serum which had been prepared against the parent Park 8 strain.

When it was observed that this 225-day-old broth culture yielded both large and small colony types upon plating, the contents of the flask were filtered through a sterile Berkefeld N filter employing a negative pressure of 9 cm. of mercury. There was no evidence of bacterial growth ever taking place in the filtrate, as the filtrate remained perfectly clear for a period of about 5 months at room temperature. Various portions of the filtrate were transferred to fresh infusion broth and incubated at 37°C. but these failed to show any evidence of bacterial growth during the 5 months' period. At various times several drops of the clear filtrate were transferred to infusion agar plates and incubated at 37°C. but these also failed to show evidence of bacterial growth as long as they were held under observation. On various occasions several drops of the clear filtrate were transferred to infusion agar plates and after a few days incubation at 37°C. the surface of the plates was washed off with sterile infusion broth and the washings transferred to another fresh infusion agar plate by the Hauduroy technic. These serial plate washings also failed to bring forth bacterial growth. From this experiment it was concluded that the organisms in neither the large nor the small colony forms contained in the 225-day-old culture were filterable.

Another series of broth tubes, 9 in number, were inoculated with a single cell culture derived from one of the Park 8 cultures (intermediate type) and, after incubation, the cultures were allowed to age at room temperature and in the dark. About once

a month sterile distilled water was added aseptically to the tubes to replace that lost by evaporation. From time to time, 3 drops of the aging culture were transferred to the surface of a fresh, sterile, infusion agar plate and the inoculum spread over the surface of the plate with a sterile glass spreader. Viable organisms were isolated from the cultures after they had aged 483 days but not after 607 days. Upon one occasion one of the tubes yielded SR, R and the small colony forms. A small colony was isolated and verified as a small colony variant of the diphtheria organism. The culture appeared to be identical with the O3–64 small colony variant.

Upon numerous other occasions small colonies have been encountered upon plates along with the larger size diphtheria colonies.

MACROSCOPIC APPEARANCES OF THE DWARF TYPE COLONIES

On infusion agar. The dwarf type colony is very much smaller than the S, SR or R types. The size of the individual colonies ranges from those colonies barely visible to the naked eye to colonies 0.2 mm. in diameter. They are thus about one-tenth to one-thirtieth the diameter of the S or *mitis* colonies. The size of the colonies does not increase appreciably upon aging nor by repeated cultivation on satisfactory culture media. The colonies are visible to the naked eye if the plate is examined carefully under proper conditions of light. The colonies are convex, round and the margins even. By transmitted light the colonies are opaque.

On potassium tellurite chocolate agar. The colonies have the same general appearance as on plain infusion agar. At first they are colorless or grayish, sometimes becoming small black specks as the culture ages, at other times failing to reduce the tellurite.

In infusion broth. The small colonies have been observed to grow in this medium with a faint turbidity which is slightly granular. They have never been observed to grow in the form of a pellicle, which is so characteristic of the SR type, nor as a gran-

ular sediment with clear supernatant, so characteristic of the R type. They have given uniform suspensions in physiological salt solution.

PRODUCTION AND DESCRIPTION OF THE G (GONIDIAL) COLONY TYPE

Growth was obtained from filtrates of diphtheria cultures at various times during these studies and under various circumstances. Since the bacterial growth fulfilled the criteria for filterability and the G culture phase, the colonies have been designated as G colonies.

1. *Isolation of G colonies from a highly acid broth tube.* In the experiment reported in table 1 the pH 5.6 tube showed no evidence of growth, whatsoever, during the 115-hour period of incubation. When three drops from this tube were transferred to a glycerol agar plate, pH 7.2 and incubated three days, a large number of very small colonies appeared. This was the first instance in which a "culture," which showed no visible evidence of bacterial growth, yielded numerous, very small colonies upon plating.

The pH 5.7 glycerol broth tube in the same series had shown, after 115 hours incubation, a very small speck of growth floating on the surface. When three drops from this tube were transferred to glycerol agar, pH 7.2, only three colonies of normal size appeared among many very small colonies. The contents of the tube were then filtered through a sterile Berkefeld N filter. This tube was chosen because it contained both the large and small colony forms. No bacterial growth appeared in the filtrate as long as it was kept under observation. Likewise, no growth occurred in serial broth 'cultures' made from the filtrate. After the filtrate had aged three weeks, two drops were seeded onto the surface of a glycerol agar plate. This plate showed no signs of growth after 4 days' incubation, at which time the surface was washed off with a few drops of sterile glycerol broth and the washings transferred to a second sterile glycerol agar plate, by the Hauduroy technic. This second plate, after three days' incubation, showed numerous very small colonies. These small colonies were called "G" colonies by Dr. Philip Hadley.

Portions of the filtrate were sealed off in ampoules and these failed to give any evidence of growth when similarly treated two years later.

Some of these small colonies were picked off to glycerol broth and to a glycerol agar slant. The growth in broth was carried along for 16 culture generations over a period of approximately 9 months. It always grew as a + or + + fine granular sediment with an occasional light clouding of the broth. At this point a broth culture was sealed in an ampoule, and when opened one year and eight months later the organisms were no longer viable.

The organisms grew on the glycerol agar slants as small, raised, smooth colonies. On the 6th glycerol agar slant in the series, the largest colonies measured 324 μ in diameter. The largest colonies ever observed in subcultures of this strain were one millimeter in diameter. The organisms making up these very small colonies were gram-positive diplo-rods about one micron in length and about 0.2 micron in width. Occasionally the size of the organisms varied from cocci of about 0.5 μ to rods of the A2 type (Westbrook, 1900) which were 1.5 μ in length. When transferred to glycerol broth, the organisms grew as a somewhat viscid sediment. Upon shaking the tube, one portion of the sediment remained attached to the bottom of the tube while the remainder swirled up into the liquid in a delicate spiral. The organisms did not ferment glucose, maltose, sucrose, lactose, galactose or glycerol, using brom-cresol purple as the indicator. When the cultures were tested with the quinhydrone pH indicator, the change in pH was found to be not greater than 0.25 pH, showing that the substances were not fermented. A glycerol broth culture sealed in an ampoule remained viable for 17 months but on other occasions the strain was not viable in ampoules 15 or 20 months old. The organisms were non-pathogenic for guinea pigs.

This strain, called F1, was kept under observation for 39 culture generations, covering a period of three and one-half years, without any change from the characteristics indicated above. The strain became contaminated with a mold during the summer vacation and all subcultures from previous transplants on hand

failed to grow. For this reason, observations on this strain could no longer be continued.

No such results were obtainable with the tubes of a very alkaline reaction, namely those at the opposite end of the series cited in table 1. Serial transfer of the Park 8 strain in alkaline broth, likewise, brought about no change in colony form. This is quite in contrast with the results which Hadley, Delves and Klimek (1931) found when working with the Shiga dysentery bacillus. They observed the G type of culture to be produced in many instances in alkaline infusion broth but never in acid media.

2. *Production of G colonies by serial transfer in acid broth.* Serial transfer of the Park 8 strain, Sr phase, in acid broth brought about the production of very small colonies, as mentioned previously. Plating out the growth from the 8th tube in the acid series, which contained 5 ml. of pH 6.2 broth plus two drops of sterile N/10 HCl onto a glycerol agar plate showed about 10 per cent of the colonies to be of the extremely small type. Several of these small colonies were fished by means of the low power of the microscope and sterile glass threads to tubes of pH 6.2 glycerol broth. Half of the subcultures showed visible growth; the other half did not. One of these subcultures which showed no visible growth in 5 days was plated out and, surprisingly, showed numerous small colonies. These small colonies, when subcultured to broth, imparted a + + clouding of very fine particles in 48 hours. The broth culture was then filtered through a Berkefeld N filter, employing about 10 cm. negative pressure. The same day the filtration was made, December 5, 1930, two drops of the filtrate were seeded onto an agar plate. No growth appeared on this series of plates carried on by the Hauduroy technic. When the filtrate was replated on February 16, 1931, numerous G colonies appeared on the plate.

One of the above subculture tubes which had showed visible growth was plated and the plate showed both the large and small colony types. One of the very small colonies was transferred to broth, incubated and replated. This plate also showed large and small colonies. One of the small colonies was trans-

ferred to pH 6.2 broth and after 48 hours incubation showed a pellicle, clouding and sediment. This culture was then filtered through a Berkefeld N filter with a negative pressure of less than 10 cm. mercury. The culture before filtration was obviously a mixture of the large and small colony types. The filtrate, itself, did not show any visible signs of bacterial growth but on the second serial Hauduroy plate from the filtrate, G colonies were present. This strain, F3, was carried through 60 culture generations over a period of over 8 years without its reversion to the original large colony type. When the strain was grown for 96 hours ($+ +$ clouding) in pH 7.3 broth to which had been added one per cent of glucose, maltose and sucrose, respectively, there was an increased acidity of 0.65 pH. In the case of lactose, galactose and glycerol, the change in pH was only 0.2 (determined by the quinhydrone pH indicator). The organisms were not pathogenic for guinea pigs.

3. *Isolation of G colonies from diphtheria toxin.* In two instances in which diphtheria toxin had been prepared by filtering 8-day-old cultures of the Park 8 strain through sterile Chamberland L3 filters, under less than 10 cm. negative pressure, G colonies were obtained by serially culturing the filtrates on glycerol agar plates according to the Hauduroy technic. In one instance the G colonies appeared on the first plate, and in the other case the G colonies appeared on the 8th plate.

4. *Production of G colonies by growth of the Park 8 strain in the presence of LiCl.* The Park 8 strain was propagated in serial passage for 15 culture generations in glycerol broth containing 0.25 per cent LiCl. The concentration of LiCl was then increased to 0.5 per cent. On the plate inoculated from the second 0.5 per cent LiCl tube there were numerous large and small colonies. Similar small colonies had appeared on earlier plates but they had been ignored since a search was being made particularly for rough colonies. Some of these small colonies were transferred to tubes of plain infusion broth in which some of the colonies produced visible growth and some did not. One of the tubes showing visible growth was seeded into a large tube of broth, incubated overnight and then filtered through a Berkefeld

N filter employing a negative pressure of 10 cm. of mercury. This filtrate, F4, was allowed to age 5 weeks and then three drops were seeded onto a glycerol agar plate. Upon incubating, this plate developed numerous very small colonies composed of small bi-polar staining rods about one micron in length. Some of these small colonies, GF4 strain, were suspended in broth and then transferred to various media, as follows:

A tube of glycerol broth, after inoculation and incubation, was sealed as an ampoule. Upon opening this ampoule two years and three months later, no visible organisms were evident.

A tube of infusion broth was inoculated and transfers made weekly to tubes of fresh broth for 27 cultural generations. Throughout the series the organisms grew in the broth as a slight turbidity with a ropy sediment. Occasionally, plates were inoculated throughout the series, but these showed only pin point colonies, about 0.2 to 0.3 mm. in diameter. The size of the cells still remained very small, being about 1.6 micron in length.

Three guinea pigs were next inoculated with the growth from the first and second broth tubes in the series. The ropy sediment was collected from the bottoms of the tubes by means of a sterile Pasteur pipette, centrifuged in a Hopkin's tube to determine the volume of the sediment and then resuspended in sterile saline. It was estimated that the suspension contained at least two billion organisms per ml.

The three guinea pigs were treated as indicated in table 2.

Another guinea pig was inoculated April 25, 1931, from the nineteenth cultural generation in the infusion broth series with all of the three-day growth from a plain agar slant. This animal appeared quite normal on May 4th, but developed paralysis within two months and died August 3rd.

A plain agar slant was inoculated and the strain propagated for 22 cultural generations. The organisms grew throughout the series as moist, pin-point colonies, about 0.2 mm. in diameter. Near the end of the series they assumed a creamy color. All of the growth from the twentieth agar slant in the series was inoculated into a 405-gram guinea pig on April 25. The animal appeared perfectly normal on May 4, but died on July 23. Like

the four other guinea pigs inoculated with this strain, the animal developed a paralysis of the posterior extremities prior to death.

Infusion broth containing one per cent glucose was inoculated and serial transfers made for 17 cultural generations. The organisms grew as a slight turbidity and a ropy sediment. The appearance and the amount of growth in the glucose broth series was practically the same as in the infusion broth series, so the series was discontinued. The last culture in the series was inoculated into a tube of infusion broth, incubated until there was good growth and then sealed as an ampoule. There were no viable organisms in the ampoule when opened three and a half years later.

TABLE 2

Effect produced in guinea pigs by the inoculation of the G74 strain

DATE		GUINEA PIG 4	GUINEA PIG 5	GUINEA PIG 6
2/ 9/31	Weight in grams	280	315	300
2/ 9/31	Intraperitoneal injection	0.5 ml.	1.5 ml.	3.0 ml.
2/17/31	Weight in grams...... . ..	315	355	310
2/17/31	Intraperitoneal injection ..	0.5 ml.	1.5 ml.	4.0 ml.
2/24/31	All of the animals appeared normal			
3/ 3/31				Paralysis noticed
3/16/31			Paralysis noticed	

All of the animals died during the latter part of March.

Litmus lactose agar (Difco), was inoculated, since Hauduroy reported this medium very favorable for the growth of the filterable forms of *C. diphtheriae* and other bacteria. Serial transplants were made about twice each week for 16 cultural generations. At first the organisms produced acid on the medium and grew well. After the 9th transplant the growth became less abundant and very little acid was produced. All of the growth from a three-day-old slant was inoculated intraperitoneally into a 320-gram guinea pig on April 25. The animal appeared perfectly normal on May 4, but was found dead on July 13.

Loeffler's medium was inoculated, since this medium is so favorable for the growth of the diphtheria bacillus, but the serial

transfers were soon discontinued because the pin-point colonies were difficult to see on the white surface of the medium.

A blood-agar slant was inoculated and the strain propagated on this medium for 64 cultural generations over a period of three and one-half years. Growth from the 20th culture in the series was inoculated intraperitoneally into a 495-gram guinea pig on April 25. The animal appeared perfectly normal on May 1, but was very emaciated and paralyzed in the posterior extremities on July 17 and was sacrificed. No macroscopic lesions were observed at autopsy. The adrenals appeared normal. The growth from the 50th culture in the series was inoculated into a

TABLE 3

Effect of the aging of the filtrate on the appearance of small colonies

DATE OF FILTRATION, 1/27/31	FILTRATE SEEDED DIRECTLY TO:				
	Plate 1	Plate 2	Plate 3	Plate 4	Plate 5
	1/27/31	2/ 1/31	2/ 6/31*	2/11/31*	2/16/31*
Date on which surface of plates were washed and the washings transferred to fresh plates	2/ 1/31	2/ 6/31*	2/11/31*	2/16/31*	
	2/ 6/31*	2/11/31*	2/16/31*		
	2/11/31*	2/16/31*			
	2/16/31*				

* Plate showed visible growth of the G colonies.

285-gram guinea pig. The animal showed no thermal reaction and did not fail to show a steady gain in weight during a period of observation extending over three months.

One drop of a suspension of strain GF4 was inoculated into each of four large test tubes of infusion broth and incubated overnight. The cultures were then filtered through Chamberland L2, 3, 5, and 7 candles, respectively, employing negative pressure of 8 to 10 cm. of mercury. The filtrates were seeded immediately onto the surface of glycerol agar plates and every 5 days the plates were washed off with sterile broth by the Hauduroy technic and the washings transferred to the surface of fresh

glycerol agar plates. The L2 filtrate yielded small colonies on the second serial plate, 10 days after filtration. Filtrates from the L3, 5 and 7 candles yielded small colonies on the third serial plate, 15 days after filtration. Of special interest was the relationship of aging of the filtrate to the rapidity of the appearance of the small colonies on the plates. Table 3 summarizes the results, which are a confirmation of the results obtained by Miss Richardson, as reported by Hadley, Delves and Klimek (1931).

MORPHOLOGY AND TINCTORIAL PROPERTIES OF ORGANISMS IN THE G TYPE COLONY

In morphology the organisms in the G colonies were short, slender rods. Sometimes the cells were nearly spherical, at other times cocco-bacillary. The organisms were granular but swollen and "involution" forms were not observed. The organisms did show pallisade arrangements. Delicate skeins or filaments were not observed but mention has been made already of the fact that the organisms grew quite often as a ropy sediment.

The organisms were gram-positive, about that there was no doubt, but many of the organisms were easily decolorized by alcohol so that in each field one saw, in addition to the typically gram-poisitive organisms, gram-negative cells and gram-negative cells containing gram-positive granules. The organisms stained unevenly with Loeffler's methylene blue stain and Neisser's granule stain.

BEHAVIOR OF THE ORGANISMS OF THE SMOOTH, INTERMEDIATE, ROUGH AND DWARF COLONIES IN SOLUTIONS OF NaCl OF VARIOUS CONCENTRATIONS

Technic. The technic for the salt agglutination test was based on that used by Wilson (1930) in his study on *Salmonella aertrycke.*

In these studies the organisms were grown on plain infusion agar slants, or in Roux flasks containing infusion agar, for 24 hours, and washed off with about 5 ml. of distilled water; 0.2 ml. of the suspension was dispensed into each of nine agglutina-

tion tubes containing 0.8 ml. amounts of distilled water, 0.2, 0.5, 0.8, 1.0, 2.0, 3.0, 5.0 and 8.0 per cent NaCl, respectively. The contents of the tubes were well mixed by shaking, then incubated in the water bath at 55°C. for 24 hours with readings at the end of one hour, four hours and 24 hours. Very seldom was there any marked change in the readings made after the one-hour incubation period. The amount of agglutination in each tube was recorded as from $+ + + +$ to $+$.

Table 4 summarizes the results obtained in a typical salt agglutination test.

TABLE 4

Salt-stability of organisms of the various colony types of diphtheria bacilli

STRAIN	COLONY TYPE	CONTROL WATER	CONCENTRATIONS OF SALINE ADDED, PER CENT							
			0.2	0.5	0.8	1.0	2.0	3.0	5.0	8.0
Escherichia coli:										
P3 (control)..	Smooth	−	−	−	−	−	−	−	−	−
P94 (control)......	Rough	−	−	++	+++	++++	++++	++++	++++	++++
C. diphtheriae:										
O3–64.......	Smooth	−	−	−	−	−	−	−	−	−
O3–64......	Dwarf	−	−	−	−	−	−	−	−	−
II...........	Intermediate	−	++++	++++	++++	++++	−	−	−	−
II...........	Rough	++++	++++	++++	++++	++++	++++	++++	++++	++++
L8	Intermediate	+++	+++	+++	+++	+++	+++	+++	+++	+++

$+ + + + =$ organisms completely agglutinated, $- =$ no agglutination of the organisms.

Summary. It can be seen from the foregoing table that the behavior of the organisms in salt solutions of various concentrations of NaCl is closely related to the manner of growth in broth. The S colony phase was not agglutinated by sodium chloride in any concentration. The rough colony phase agglutinated spontaneously in distilled water and sodium chloride solutions of all concentrations. The intermediate colony phase showed partial agglutination in all concentrations of NaCl or in the lesser concentrations only. The *mitis* and *gravis* colony forms were found to be similar to the S and SR culture phases, respectively.

PATHOGENICITY OF THE ORGANISMS IN THE SMOOTH, INTER-MEDIATE, ROUGH AND DWARF COLONY PHASES

Technic for pathogenicity test. Organisms to be tested for pathogenicity were inoculated over the entire surface of suitable solid media, usually blood agar, incubated 24 to 48 hours and then washed off the media with small amounts of sterile infusion broth. The growth from one blood-agar slant was injected subcutaneously or intraperitoneally into each guinea pig of approximately 250 grams in weight. Usually a control guinea pig which had received at least 1000 units of diphtheria antitoxin was injected at the same time.

Results. Intraperitoneal injections of the S, SR, R and D forms proved fatal. Injections of the G forms brought about paralysis and death only after a period of several weeks, as previously mentioned. Control animals with antitoxin were not inoculated with the G cultures.

Upon two occasions avirulent variants were isolated from cultures of a Park 8 strain. About 25 broth cultures in test tubes of a Park 8 strain producing SR colonies were sealed off as ampoules after the cultures had shown good growth, usually after 48 hours incubation. These ampoules were stored at room temperature and in the dark. From time to time an ampoule was opened and a few drops of the contents transferred to the surface of a fresh agar plate. In the case of ampoule 096, which had aged 140 days, only one colony appeared on the surface of a glycerol agar plate. This colony was white, moist, round, convex and shiny, decidedly smoother than the colony type of the strain at the time it was sealed in the ampoule. The organisms were agglutinated by anti-Park 8 serum, produced the *"mitis"* type of colony on potassium tellurite chocolate agar but failed to kill guinea pigs weighing 285 and 369 grams, respectively.

Of greater interest is another variant of the Park 8 strain which was produced under similar conditions. Ampoule 097 was opened after 132 days and a few drops spread over the surface of a glycerol agar plate. Four colonies appeared on this plate. The colonies were distinctly smoother than the colonies produced

at the time the Park 8 culture was sealed in the ampoule. One of the colonies was transferred to a glycerol agar slant and maintained on either glycerol agar or blood agar slants as variant strain 097. The organisms gave the typical fermentations of the parent Park 8 strain, *i.e.*, acid from glucose and dextrin and no action on sucrose, and were agglutinated by anti-Park 8 serum. On December 6, 1933, the entire 48-hour growth from the surface of a blood agar slant was inoculated subcutaneously into a 574-gram guinea pig. The animal remained well. The test was repeated on January 13, 1934, using a guinea pig weighing 240 grams and this animal remained normal. It was concluded that these two variants of the Park 8 strain, 096 and 097, respectively, represented avirulent forms.

On November 20, 1934, a 326-gram guinea pig was inoculated with all the 24-hour growth from a blood agar slant of 097 and remained normal. On November 27, 1934, when the animal received a second and similar dose, the animal weighed 402 grams. The animal remained normal for ten days but was found dead on December 18 (20 days). On December 8, 1934, the 12th generation of the strain was inoculated intraperitoneally into two guinea pigs weighing 278 and 282 grams respectively, and both animals were dead within 48 hours.

This experience suggests that the Park 8 strain of the diphtheria bacillus became avirulent upon aging in ampoules but regained its virulence, as judged by guinea pig inoculations, after propagation on ordinary blood agar slants.

TOXIGENICITY OF THE ORGANISMS IN THE SMOOTH, INTERMEDIATE, ROUGH AND DWARF COLONY PHASES

Technic for toxigenicity test. For testing the toxigenicity of a culture, the organisms were grown for 7 to 10 days in a Roux flask containing about 100 ml. of infusion broth, pH 7.8, containing Bacto-proteose peptone. The flask was shaken to break up the pellicle, allowed to stand for a short time to allow the large clumps to settle to the bottom and the supernatant liquid decanted to a sterile filter assembly. Filtration was made through either Berkefeld or Chamberland filters by a technic which has

been described in detail elsewhere (Morton, 1938). The filtrate was placed in suitable sterile containers, a preservative, 0.3 per cent tricresol, added, and then placed in the cold room or refrigerator for a week or 10 days. After aging, the filtrate was injected in various amounts into normal 250-gram guinea pigs. The smallest amount of sterile filtrate killing a 250-gram guinea pig in 96 hours was taken as the minimum lethal dose (M.L.D.) of the filtrate.

Results. The S, SR, R and D forms elaborated a soluble toxin, as evidenced by the fact that a sterile cell-free filtrate of the cultures produced death in guinea pigs. There was, however, a quantitative difference, the D forms being much less toxigenic. Toxins from the S, SR and R forms had an M.L.D. of about 0.02 ml., whereas 1 ml. of a similar filtrate from the D form killed a 240-gram guinea pig on the eighth day. Filtrates of the G forms were not tested for toxicity since it was not possible to prepare toxin under the usual standard conditions with this culture type.

The toxin produced by two serological types was neutralized by commercial antitoxin.

AGGLUTINATION, OR AGGLUTININ ABSORPTION, OF ORGANISMS IN THE SMOOTH, INTERMEDIATE, ROUGH, DWARF AND GONIDIAL COLONY PHASES

Technic for agglutination test. Whenever comparative tests were made the original dilution of serum in saline was prepared in sufficient amount to serve in all of the tests. The concentration of the NaCl solution was varied so as not to cause spontaneous clumping of the strains. Usually 1 per cent NaCl was employed. Saline and normal serum controls were included. The rack of tubes was placed in a water bath at 55°C. for 4 hours and then kept at room temperature overnight. Readings were made as soon as the tests were set up, after one and four hours and also after the tubes had stood overnight.

Technic for agglutinin absorption. A measured amount of serum was absorbed with generous quantities of packed, living organisms in a graduated centrifuge tube at 37°C. on three oc-

casions, which was found to be sufficient when working with sera of a titer not over 1:5120. It is not as easy as with many other organisms to produce agglutinating sera in rabbits.

Results. This part of the work has thus far only been carried far enough to establish that there are serological relationships between the several variants. The question of whether or not serological differences exist is reserved until more comprehensive experiments have been made.

The S, SR and D variants of the Park 8 strain have all been agglutinated in Park 8 antiserum to similar titer.

The two G (gonidial) strains which were tested after they had become large enough to be grown in sufficient amounts for antigens, were not found to be agglutinated by serum prepared against the parent culture.

It was impossible to employ the R variants for agglutination because of the tendency to spontaneous agglutination. However, the rough variant of the no. II strain absorbed all the agglutinins from the immune serum prepared by immunizing rabbits against the smooth form of no. II strain.

HEMOLYTIC ACTIVITY OF ORGANISMS IN THE SMOOTH, INTER-MEDIATE, ROUGH, DWARF AND GONIDIAL COLONY PHASES

Technic for hemolysis test. Defibrinated rabbit or human blood was mixed with 10 volumes of saline and centrifuged. The supernatant fluid was pipetted off and another 10 volumes of saline added, thoroughly mixed and the suspension recentrifuged. The supernatant fluid was pipetted off and the washed blood cells resuspended in saline to make a 5 per cent suspension. For the test, itself, 0.5 ml. of fresh 5 per cent suspension of washed red blood cells was mixed with 0.5 ml. of a 48-hour broth culture of the organisms and the mixture incubated in a water bath at 37°C. for two hours. A reading was then made, the tubes kept overnight at room temperature and the final reading made next morning.

Table 5 summarizes the results obtained by testing the various colony types against the washed red blood corpuscles of rabbit and human origin.

The results shown in table 5 indicate that the greatest hemo-

lytic power is associated with the S and Sr colony types. The extreme rough colony type is slightly hemolytic and the D colony type non-hemolytic. This might account for the variations in hemolytic power of diphtheria cultures obtained by earlier workers as very little, if any, attention was paid to the colony form of the strains when tested for hemolysis.

TABLE 5

Results of testing members of the diphtheria group and different colony types of the same strain of C. diphtheriae against red blood cells

STRAIN AND COLONY TYPE	RABBIT RED BLOOD CELLS	HUMAN RED BLOOD CELLS
0.85 per cent saline (control)........	—	—
Streptococcus hemolyticus (control)...	++++	++++
Streptococcus viridans (control)...	A	A
Corynebacterium hofmanni (control).	—	—
Corynebacterium xerosis (control)	—	—
C. *diphtheriae*, O3–64 "S".	++++	++++⁻
C. *diphtheriae*, O3–64 "D"....	—	—
C. *diphtheriae*, II "Sr"	++++	++++
C. *diphtheriae*, II "R"	++−	±
C. *diphtheriae*, II "S" derived from II "R".	+++	++
C. *diphtheriae*, L8 "Sr".	+++	++
C. *diphtheriae*, L8 "D"	—	—
4 strains C. *diphtheriae* "*mitis*"	++++	++++
2 strains C. *diphtheriae* "*gravis*".	++	±
2 strains C. *diphtheriae* "*gravis*"	+	±
5 strains C. *diphtheriae* "G"...	—	—

      — = no change in the appearances of the red cells.
      ± = doubtful change in the appearances of the red cells.
  ++++ = complete hemolysis (beta type) of the red cells.
 ++++⁻ = not quite complete hemolysis but more than +++, etc.
      A = Alpha type of hemolysis.

FERMENTATION REACTIONS OF ORGANISMS IN THE SMOOTH, INTERMEDIATE, ROUGH, DWARF AND GONIDIAL COLONY PHASES

All the diphtheria cultures tested for fermentation reactions, and this included *gravis* and *mitis* strains as well as S, SR, R and D strains, were found to produce acid from glucose and dextrin but not from sucrose. The S, SR, R and D colony types had the same fermentation reactions. There was, however, slight variation in the rate at which the acid was produced. The

SR appeared to produce acid slightly faster than the S form, but the difference was slight. There was a noticeable difference between the rate of acid production of the R as compared with the S and the SR strains, the R being much slower. The D type produced acid very much more slowly than the S, SR and R strains. The important point is that the S, SR, R and D colony variants of a strain have the same qualitative fermentation reactions, there being only difference in the rate in which acid is produced.

The G strains which were tested for fermentation reactions either failed to produce acid or else produced only a questionable amount.

RESISTANCE TO HEAT AND COLD OF ORGANISMS IN THE SMOOTH, INTERMEDIATE, ROUGH, AND DWARF COLONY PHASES

Heat. Text-books vary considerably in their statements as to the thermal death point of *C. diphtheriae.* It is generally stated that broth cultures of the diphtheria bacillus are killed by ten minutes exposure at a temperature of 58° to 70°C. One observes numerous statements in the literature which imply that the rough stage of a culture is usually more resistant to unfavorable conditions than the smooth stage. To verify that point, determinations of the resistance to a temperature of 56°C. were made on 24-hour-old broth cultures of several strains of the diphtheria bacillus as well as on different variants of the same strain.

Technic for thermal death point determination. Sterile Pasteur pipette stock was drawn into long capillary tubes of about one millimeter inside diameter and of uniform bore and walls. After flaming the tip of the capillary and allowing it to cool, the bacterial suspension was allowed to flow up into the tube by capillary attraction to a height of one inch. Air was then drawn into the capillary tube until the 1-inch column of bacterial suspension reached the 2-inch mark, then the end of the capillary was sealed in a gas flame. The capillary tube was then broken off one inch above the bacterial suspension and that end sealed in the gas flame. The sealed capillary tube, which contained a column of bacterial suspension in the middle with a column of air at

each end, was dropped into disinfecting solution to destroy any organisms which might be on the surface. A series of tubes prepared in this manner were then placed, completely submerged, in an electrically heated water bath at 56°C. and at various intervals of time a tube was taken from the water bath, immersed for a few seconds in tap water at room temperature and then placed in a rack. After the last tube in the series had been removed from the water bath the contents of each tube were transferred to a tube of sterile broth. This was accomplished by wiping the outside of the capillary tube with alcohol, allowing the

TABLE 6

Thermal death point determinations of several strains and different colony types of C. diphtheriae

STRAIN	COLONY TYPE	CONTROL	TIME IN MINUTES AT 56°C.					
			1	2	3	4	5	6
C. diphtheriae, L8	Sr	+	+	−	−	−	−	−
C. diphtheriae, L8	D	+	+	−	−	−	−	−
C. diphtheriae, O3	Sr	+	+	+	−	−	−	−
C. diphtheriae, O3	D	+	+	+	−	−	−	−
C. diphtheriae, II	Sr	+	+	+	+	−	−	−
C. diphtheriae, II	R	+	+	+	+	−	−	−
C. diphtheriae, O5	Sr	+	+	−	−	−	−	−
C. diphtheriae, IX	S	+	+	+	+	−	−	−
C. diphtheriae, XII	Sr	+	+	+	+	−	−	−
C. diphtheriae, VIII	S	+	+	+	−	−	−	−
C. diphtheriae, XI	Sr	+	+	+	−	−	−	−

+ = growth.
− = no growth.

alcohol to evaporate, breaking one end of the tube close to the column of bacterial suspension and forcing the suspension into a tube of sterile infusion broth by gently warming the end of the capillary tube which contained the entrapped air. The tubes inoculated with the heated suspension were incubated for 96 hours, with readings being made at the end of each 24-hour period. Usually four series of tubes were carried through the operations at one time, so that all the factors in the experiment except the culture under test were as uniform as possible.

Results. Table 6 summarizes the results obtained in the ther-

mal death point determination of various diphtheria strains and also of the various colony forms of the same strain.

It can be seen from this table that the diphtheria bacillus is not very resistant to heat, being killed by one to three minutes exposure at 56°C. The slight variations in resistance to heat appear to be associated with the individual strains rather than with the colony forms of the strains. These findings are not in agreement with those of Weiland and Leinbrock (1938).

Cold. Since it is fairly generally known that the diphtheria bacillus is less effected by cold than by heat it was decided to try the S and R variants of the same strain against low temperature for varying periods of time. A knowledge of resistance of diphtheria bacilli to cold is also essential for the preservation of the organisms, since recent trends in the preservation of bacterial cultures are to employ methods of drying cultures from the frozen state.

A freezing mixture of solid carbon dioxide (Dry-Ice) and methylcellosolve was employed. This freezing mixture gave a temperature of -60 to $-75°C$. One milliliter of bacterial suspension was deposited in separate sterile Pyrex glass vials. Estimation of the number of organisms in the bacterial suspensions used was made by means of agar pour plates. The Pyrex glass vials containing the suspension were immersed in the freezing mixture and after varying periods of time two vials (one containing smooth organisms and the other containing rough organisms) were removed. After the suspensions had thawed out at the room temperature, 15 ml. of agar, melted and cooled to 45°C., were added to each vial, the contents were well mixed and poured into a sterile Petri dish. It was not necessary to make serial dilutions of the suspensions after being frozen and thawed as the number of viable bacteria had been greatly reduced. The number of organisms surviving the freezing and thawing, as evidenced by the number of colonies on the agar plates, were counted and the percentage of destruction calculated (table 7).

It can be seen from the results described in this section that the rough phase of the diphtheria bacillus was no more resistant to cold under these circumstances than the smooth phase.

RESISTANCE TO AGING OF ORGANISMS IN THE SMOOTH, INTERMEDIATE, ROUGH AND DWARF COLONY PHASES

During the course of the studies many of the cultures were inoculated into tubes of infusion broth, incubated (usually overnight) until there was good growth, and the tubes were then sealed as ampoules. It was found that the diphtheria bacillus did not live as long in the ampoules as did many other organisms. Test tubes of practically the same diameter were used and they con-

TABLE 7

Destruction of C. diphtheriae by low temperature

STRAIN	DESTRUCTION IN MINUTES AT −75°C.					
	2	5	10	20	30	60
	per cent	per cent	per cent	per cent	per cent	per cent
C. diphtheriae, II, S.	76.6	77.3	79.8	83.8	74.5	77.1
C. diphtheriae, II, R	76.8	76.3	77.8	84.3	85.7	80.7

TABLE 8

Resistance of the various colony types of C. diphtheriae to aging in ampules

STRAIN	COLONY TYPE	ALIVE*	DEAD
		days	days
C. diphtheriae, II	Very nearly S	90	107
C. diphtheriae, II	R	224	267
C. diphtheriae, O3	SR	171	231
C. diphtheriae, O3..................	D	467†	

* This time represents the maximum number of days in which the organisms were ever found viable.

† End of series.

tained practically the same amount of infusion broth. Usually 15 or more tubes were employed in a series. Each tube in a series was inoculated with the same size inoculum. The ampoules contained approximately 5 ml. of culture and about the same volume of air above the cultures. The ampoules were stored at room temperature and in the dark. From time to time an ampoule was opened and some of the contents was seeded to an agar plate. Whenever growth occurred it was definitely identified as *C. diphtheriae*. Table 8 summarizes the results.

It can be seen in this experiment that there was a distinct difference in the resistance to aging between the colony forms of the same strain.

REDUCTION OF NITRATES

Contrary to Bergey's Manual (1939) diphtheria cultures produced nitrites when grown in the presence of KNO_3.

CHROMOGENESIS

Various observers have reported pigmentation in certain diphtheria cultures. It has been our experience during these studies that chromogenesis took place seldom and irregularly. Upon different occasions throughout these studies it was noticed that certain strains of diphtheria bacilli developed a pigment which was somewhat out of the ordinary. A lemon-yellow pigment was observed in several cultures of one of the Park 8 strains which was in the Sr phase. In one instance the pigment was produced in a stock culture of the Park 8 strain being carried on Loeffler's medium and in the cold room. This pigmentation appeared in a few generations after receiving the strain. In another instance the lemon-yellow color developed in a single-celled strain of the Park 8 culture kept on plain infusion agar at room temperature and in diffuse daylight. It appears that the production of pigment is not necessarily associated with a change in any other characteristic of the culture. The pigmentation seems to appear more often and to a greater extent in the S or Sr culture phase.

CELL MORPHOLOGY

More significance is attached to the morphology of diphtheria organisms than in the case of any other microorganism; yet the diphtheria organism is most pleomorphic. Since attention has been directed to the study of bacterial colonies, it has been found that certain morphological forms can be associated with the various colony types. This is also true in the case of the diphtheria species.

Organisms from the S colony are fairly long and slender, sometimes slightly bent and showing the typical V, L and pallisade

arrangements. They show the classical irregular staining and granules by methylene blue. They are gram-positive.

Organisms from the SR colony are longer than the organisms from the S colonies. They show a greater tendency toward irregular staining and irregularly swollen forms. They show the typical arrangements and are gram-positive.

Organisms from the R colony are characterized by the definite tendency for the organisms to form chains or threads. They are gram-positive and usually stain more intensely than do organisms from the other colony types. The cells are usually thicker than the organisms in the S and D colony types.

Organisms from the D colonies are very short, and somewhat thick, rods. They are gram-positive and usually stain solidly with the gram and methylene blue stains. They show the typical arrangements of cells and some tendency towards the irregularly swollen forms.

Organisms from the G colonies are short rods, sometimes being nearly spherical. They show the typical arrangement of cells, are variable towards the gram stain and stain irregularly with methylene blue.

MORPHOLOGICAL FORMS NOT ASSOCIATED WITH COLONY FORM

A. Coccoid forms.[3] At various times during these studies the bacilli were observed to assume coccus-like appearances. It was found possible to cause this change to appear or disappear at will. The change, either from rods to coccoids or *vice versa*, was in many cases practically complete. "Coccus," "coccal," "coccoid" and "staphylococcus" forms of the diphtheria bacillus have been described in the literature. Many of the circumstances bringing about these forms have been listed elsewhere (Morton).

The coccoid forms were encountered under the following conditions: In the course of the isolation of a single cell of the Park 8 strain by the method of Kahn (1929), a very rich medium was employed, consisting of infusion broth, pH 7.6, containing 5 per cent glycerol to which was added sterile glucose solution and

sterile normal rabbit serum to the concentrations of one and 20 per cent, respectively. The single cell picked was of Wesbrook's type C. Culturally and serologically the single-cell and parent strains were identical. When the single-cell strain was grown in this rich medium, only spherical cells were present; the organisms appearing very similar to staphylococci. When these spherical cells were transferred to ordinary blood agar, typical diphtheria bacilli resulted.

The single-cell culture showing typical diphtheria bacilli was seeded into glycerol-glucose-serum broth and after 24 hours the growth showed only an occasional granular rod, a few short, plump, solid-staining rods and numerous spherical cells or slightly elongated spheres. Seeded simultaneously into glycerol-glucose-serum broth and onto a Loeffler slant the resulting cultures after 48 hours incubation showed spherical cells and typical rods, respectively. The culture on Loeffler's medium contained granular, barred and solid-staining rods, whereas on blood infusion agar and glycerol agar, the culture showed the granular type of rods almost entirely.

To ascertain, if possible, which ingredient in the glycerol-glucose-serum broth was producing these variations in morphology, the following media were seeded from a Loeffler slant culture and incubated 48 hours.

Infusion broth, pH 7.3
Infusion broth + 20 per cent serum
Infusion broth + 1 per cent glucose
Infusion broth + 1 per cent glucose + 20 per cent serum
Infusion broth + 5 per cent glycerol
Infusion broth + 5 per cent glycerol + 20 per cent serum
Infusion broth + 5 per cent glycerol + 1 per cent glucose
Infusion broth + 5 per cent glyerol + 1 per cent glucose + 20 per cent serum

The media are listed according to their ability to change the rod forms into coccoid forms, the first named medium being the least effective and the last named, most effective. Ten-per-cent normal rabbit serum could be substituted for the 20 per cent with about the same results. Stained preparations were made

after the incubation period (fig. 1), then a subculture was made from each type of medium onto a Loeffler slant. The cultures on Loeffler's slants were examined after 48 hours incubation and in all cases the morphology was found to be identical with that of the Loeffler culture of the stock strain.

To determine the effect of normal serum of different species of animals on the morphology of the diphtheria bacillus, the following media were inoculated from a blood agar slant culture and incubated 48 hours.
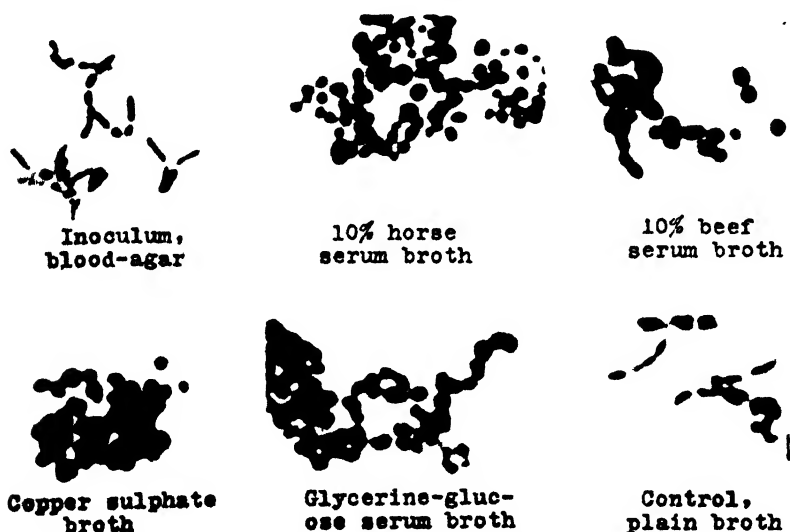
FIG. 1. C. DIPHTHERIAE, PARK 8, SINGLE CELLED STRAIN, GROWN 48 HOURS AT
37° C.
Methylene blue stain, × 2650.

Infusion broth + 20 per cent guinea pig serum
Infusion broth + 20 per cent rat serum
Infusion broth + 20 per cent human serum
Infusion broth + 20 per cent rabbit serum
Infusion broth + 20 per cent horse serum
Infusion broth + 20 per cent beef serum

Practically no change in the morphology of the culture was noticed in the case of the guinea pig serum. Rat and human sera produced a slight change to the solid-staining rod and coccoid

forms, there being no difference in the effect of serum from
Schick + and Schick − individuals; rabbit serum caused a dis-
tinct change to the coccoid and solid-staining forms while the
horse and beef sera produced a very marked change to the coc-
coid forms.

No agglutinins could be demonstrated in the sera which caused
the production of coccoid forms. Normal horse serum was
separated into its albumin and globulin fractions by the addition
of an equal volume of saturated solution of ammonium sulfate.
After filtration, dialysis and sterilization by Berkefeld filtration
the albumin and globulin fractions were added to the culture
medium. When either fraction was added to the culture medium,
coccoid forms resulted.

Best coccoid formation was obtained when the sera were added
to the culture medium under aseptic conditions. If the serum
and medium were autoclaved, there was very little tendency for
the production of coccoid forms. Heating at 56°C. for 30 minutes
had no noticeable effect on the ability of a serum to produce the
coccoid forms. Normal horse serum from four different sources
was tried and, while the extent of coccoid formation varied,
none of the sera failed to give coccoid forms.

To test the observation of Yarisawa, that the amount of heat
employed for sterilization of the Loeffler's medium had an effect
on the morphology of the organisms, a batch of the medium
was prepared; part of it being sterilized in the autoclave as de-
scribed previously and the remainder being sterilized in the usual
manner by heating in the Arnold sterilizer on three successive
days. Both types of media were then inoculated from the same
stock culture and incubated 48 hours. The autoclaved medium
showed long, slender, pleomorphic, granular bacilli, whereas the
medium which had been sterilized in the Arnold gave shorter
and more solidly staining organisms.

In summary, it was concluded from these experiments that the
morphology of the diphtheria bacillus may be changed to that
of coccoid cells under the influence of normal sera from various
species of animals. The serum from individuals of a given
animal species varies somewhat in its ability to transform the

morphology to that of coccoid forms; the globulin and albumin fractions appear to work equally well in bringing about the transformation. There were no demonstrable agglutinins in sera which brought about the production of coccoid forms. The heating of a serum at 56°C. for 30 minutes did not alter its ability to transform the morphology of the diphtheria bacillus. Prolonged heating, such as autoclaving at 120°C. for 20 minutes, reduced, but did not completely destroy, the ability of serum to change the morphology of the diphtheria bacillus to that of coccoid forms.

To determine whether or not the "coccal" forms, which had been produced by Pope and Pinfield with the aid of copper sulphate, were in any way similar in appearance to the coccoid forms produced in glycerol-glucose-serum broth, the following experiment was performed.

Sterile beef infusion broth, pH 7.3, (sterilized in the usual manner) was dispensed into sterile test tubes in 5 ml. amounts. A 0.04 per cent solution of copper sulphate in distilled water was prepared and sterilized by filtering through a Berkefeld W filter using a negative pressure of 10–15 cm. of mercury. The sterile copper sulphate solution was dispensed in varying amounts into the tubes of broth to give final concentrations of 17 to 38 mgm. of copper per liter. To make sure that any change occurring in the morphology of the culture was due to the presence of the copper sulphate in the medium and not to dilution, a similar series of broth tubes was set up to which were added corresponding amounts of sterile distilled water. All the tubes were incubated 24 hours to insure sterility of the medium, then two drops of a suspension of the single-cell strain, prepared by washing off a blood-agar slant, were added to each tube. The tubes were then incubated 48 hours and stained preparations made.

In all the tubes containing the copper sulfate, the morphology of the organisms was that of coccoid and diplococcoid forms, with a few solid staining and barred forms present. The control series of tubes showed only typical, granular bacilli.

The stained preparations made of cultures from glycerol-glucose-serum broth and from copper sulphate broth appeared

practically identical. The fine granular types of growth in the
two media also appeared very similar. The growth from a flask
of broth containing copper in the concentration of 23 mgm. per
liter was centrifuged and resuspended in 0.85 per cent solution
of NaCl. The resulting suspension of coccoid forms was ag-
glutinated by rabbit serum produced against the granular forms
of Park 8 rods to the full titer of the serum. Transplants of these
coccoid forms were as virulent for guinea pigs as the stock strain
which was maintained on blood agar.

Pope and Pinfield attributed the "coccoid" formation as being
due to the presence of the copper ion in the medium. Some
observations suggested that sulphate and magnesium ions as well

TABLE 9

Effect on the morphology of C. diphtheriae of various salts in the medium

SALT	MORPHOLOGY OF CELLS WHEN 48 HOURS OLD
$CuSO_4$	Coccoids
$MgSO_4$	Coccoids
Na_2SO_4	Coccoids and a few very short rods
$(NH_4)_2SO_4$	Short, solid rods in pairs and some coccoids
$Cu(NO_3)_2$	Coccoids and some very short rods
$NaNO_3$	Typical rods, very granular
$MgCl_2$	Coccoids and some very short rods
$NaCl$	Typical rods

Control: 5 ml. of broth and 2 ml. of distilled water. Typical rods.

as copper ions might produce the change in morphology. The
final concentration of copper sulphate in the broth which gave
good coccoid formation was N/1000. Various other salts were
made up in aqueous solution, sterilized by Berkefeld filtration
and added in 2 ml. amounts to tubes containing 5 ml. of broth
to give a final concentration of N/1000. The plan of the experi-
ment was essentially the same as that described for the previous
experiment. Table 9 summarizes the experiment.

Cultures consisting of the coccoid forms were inoculated into
plain Hiss serum water + brom-cresol purple and also into tubes
of the same medium containing, respectively, glucose, sucrose,
and dextrin in the amounts of one per cent. The fermentation

reactions were typical for *C. diphtheriae*. As reported by Parish, the coccoid forms persisted in the Hiss serum water. When the stock culture of *C. diphtheriae* was inoculated into a similar set of tubes of Hiss serum water + brom-cresol purple containing the three carbohydrates and a control tube of plain Hiss serum water and indicator, the growth in the control tube and in the tube containing sucrose (which was not fermented) was made up of a large number of coccoid forms and short solid-staining rods. The coccoid forms were even more numerous in the glucose and dextrin tubes in which the carbohydrates had been fermented. A tube of infusion broth pH 6.2 gave small forms, but not coccoids.

In addition to the influence of the normal sera of various species of animals upon the morphology of the diphtheria bacillus, it was concluded from these experiments that the presence of very small amounts of various salts to the culture medium also had a marked effect. In extension of the views of Pope and Pinfield, who believed that it was the copper ion in copper sulphate which caused the transformation to the coccoid forms, the sulphate ion, as well, appears to be able to bring about the transformation. It appears, then, that either the anions or the cations may influence morphology. The coccoid cells appear very much alike, whether produced by the addition of various normal sera or salts to the culture medium.

Discussion. The purpose of this section of the work has been to investigate these recorded instances in which the diphtheria bacillus assumed the coccoid form so that these transitory forms will not be confused with coccus-like variants, which appear to be entirely different in nature. The term "coccoid" is given these forms because they are very transitory, and do not take on any properties that are strikingly different from the rod forms from which they are derived and to which they readily revert. Although they may appear to be perfect spheres, it is also possible to see, mixed with them, cells which are slightly elongated and which are almost impossible to distinguish as either short, swollen rodlets or slightly elongated spherical cells.

Yarisawa's (1926) conclusion that the occurrence of coccoid

forms of *C. diphtheriae* was a transitory phenomenon in accordance with conditions, variety and surroundings of the medium apparently holds true and is borne out by the experiments in this section and literature cited previously. The change from rods to coccoids, or *vice versa*, takes place in one generation and, in many cases, is practically complete. This does not parallel the production of such dissociative variants as the rough and smooth colony types, for which a number of generations are usually required and, in which the transformation is often more gradual. Another feature in which the coccoid forms differ from actual dissociants, is that the former are not stable. Toxicity tests could not be performed because as soon as the culture was placed in a plain bouillon, suitable for toxin production, the cells assumed the rod form. Even when kept in contact with the agent which brought about their formation, the coccoids gradually reverted to the rod form. There was thus no way of maintaining a pure culture of coccoid forms suitable for prolonged study.

The fact that certain animal products, when brought into contact with the culture, exert a marked change in the morphology of the culture might explain the staphylococcus forms which Killian was able to re-isolate from guinea pigs, the change in morphology which Gins and Jermoljewa and Malcherek noticed when a sterile emulsion of guinea pigs' liver or kidney was added to the medium, and the coccoid forms which Crowell observed with the peritoneal fluid of normal guinea pigs, *in vivo*. That insufficient heating does not destroy the power of animal products to alter the morphology is in agreement with the observation that Loeffler's medium gave a variety of morphological forms (Yarisawa), depending on the amount of heat to which it had been subjected during the sterilization process; that Grubb and Koser obtained coccoid forms on liver infusion agar; that Hadley obtained coccoids by means of ascitic fluid, and that occasionally the coccoid forms appear in ordinary media (Heinemann, Parish, Parker). Pope and Pinfield's observation that the presence of a certain ion may exert a profound influence on the morphology of *C. diphtheriae* was verified. The copper ion,

however, is not the only one which can bring about this change to coccoid forms. It appears that magnesium and sulphate ions may produce this change as well. This perhaps also accounts for Maver's observations of coccoids in synthetic media.

The colony type of a culture of coccoid forms remains the same as when the culture is composed of rods. The tendency of the cells of *C. diphtheriae* to adhere to one another after fission is very striking with the coccoid forms, giving diplo and tetrad forms. Maver (1931) shows some characteristic tetrad forms from digested Loeffler's serum, which are identical to the tetrad forms observed in media containing raw serum. Plain agar plates streaked from cultures showing these tetrad forms show only colonies of typical diphtheria bacilli.

With methylene blue the coccoid cells may stain solidly or, occasionally, show one metachromatic granule within the cell. By Beck's stain the majority of organisms take the counter stain while a very few of them stain solidly or partially with gentian violet.

Grubb and Koser observed rod forms to "contract" into coccoid cells on liver infusion agar. All of these observations indicate that these coccoid forms merely represent another morphological type of the diphtheria bacillus. These coccoid forms do not assume characteristics which would place the culture, necessarily, in a colony phase different from that of the rods from which they were derived.

There are, however, instances in the literature in which the change to coccus-like cells suggests a phenomenon which is different from the production of coccoids as herein described, namely, the A-forms (Pettenkofer) of Kuhn (1924), the C-forms (coccus) of Kuhn and Sternberg (1931) and the G-forms of Hadley, Delves and Klimek (1931). In these cases the culture has become changed and stabilized in such a manner as to indicate a definite culture phase.

The C-forms of Kuhn and Sternberg and the G-forms of Hadley, Delves and Klimek are now thought to be one and the same culture phase and to represent the filterable form of the organism (Hadley, 1933). The A-forms (Pettenkofer) of Kuhn have not been

explained on the basis of colony phases. In one instance when a strain of the diphtheria bacillus was being propagated in infusion broth plus as much LiCl as the organisms would tolerate and still produce visible growth, morphological forms were encountered which were suggestive of the Pettenkofer forms. These forms were not investigated further and reverted to typical rod forms during their propagation on plain agar slants.

Kuschnarjew (1930) and more recently Stone and Hobby (1934) described "coccus" forms of the diphtheria bacillus which may be different from the coccoids just described. Cultures of these "coccus" forms in broth showed turbidity, but no pellicle. The growth near the surface was ropy. Colonies on solid media were mucoid in consistency. Stone and Hobby found their "coccoid" forms to be more susceptible to bacteriophage than the rod forms. It might be that these workers were dealing with a stage of the diphtheria culture quite different from the rod forms.

B. Changes in the morphology depending upon the youth and age of a culture. A few of the early workers were of the opinion that there was a normal evolution in the morphological forms of the diphtheria bacillus. In this connection the works of Deny (1903), Clark and Ruehl (1919), Powell (1923) and Henrici (1928) may be mentioned. All of these workers found that there was a definite series of changes in diphtheria cultures when planted on fresh media and observed over a fairly long period of time. To lend further support to the findings of the workers mentioned above, the observations which were made on the Park 8 strain during these studies might be briefly mentioned.

The strain was inoculated into glycerol infusion broth and then stained preparations were made hourly for the first 24 hours (fig. 2). Additional observations were made at the end of 36, 48, 72 and 96 hours as well as at the end of one, two, three, four, five and seven weeks. During the first few (two to five) hours of growth the cells were small and solid staining, usually appearing as very short rods or as spheres. After the 5th hour the cells elongated. Granules appeared at about the 13th hour. The

length of the rods remained fairly constant from about the 22nd hour to the end of the first week; thereafter they elongated somewhat for a period of a few weeks, but at the end of the 7th week they had shortened to coccoid forms. Club forms were first observed at 24 hours. Barred forms were first noted at the end
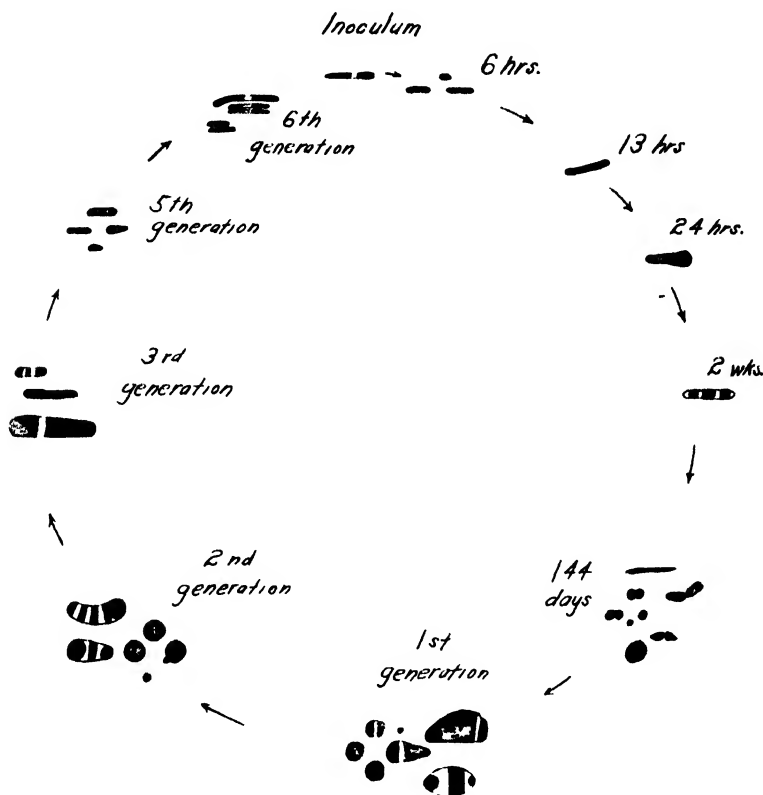
FIG. 2. A SERIES OF MORPHOLOGICAL FORMS OBSERVED TO TAKE PLACE AS A CULTURE OF THE PARK 8 STRAIN OF C. DIPHTHERIAE AGED AND AS THE AGED CULTURE RETURNED TO NORMAL GROWTH VIGOR

of the second week. About the end of the first week two general types of rods were present. One type was long and slender and stained a very faint blue with Loeffler's methylene blue, whereas the other general type of rod was short and thick and more solid staining. The granules also assumed a different appearance

when stained by methylene blue. Until the first week the granules had stained an intense blue, afterwards they were somewhat smaller and stained a bright red. These observations were made when an actively growing culture was transplanted to fresh glycerol broth and are in agreement with the observations of the workers mentioned above.

No record could be found of observations upon the series of events when an old, dormant culture was transplanted to fresh media. For these observations a broth culture of the Park 8 strain, which had aged in an ampoule for 144 days was used. Upon opening the ampoule, a Loeffler's methylene blue stain revealed that about one-half of the organisms were moderately long, slender, typical diphtheria rods of a uniform light-blue tint. The remaining rods were granular. Some of the rods possessed small granules of a deep blue color while a few of the rods contained granules of a reddish color. Sometimes these red granules appeared well separated 'from any bacterial cell and at other times some of the granules appeared to have a small amount of bluish debris adhering to them. The isolated granules varied in size from the limit of visibility to 0.5 of a micron in diameter. Occasionally the red granules gave a very clear-cut picture of a small diplococcus, suggesting that they might be undergoing division.

Three drops of this 144-day-old culture were spread over the surface of a glycerol agar plate and the plate incubated. Nothing appeared on the plate during the first 48 hours. At the end of the 5th day, about 300 well separated colonies were present on the plate. These colonies varied in size from 0.2 to 0.5 mm. in diameter. The colonies were convex, opaque, of a creamy color and the margins entire. As far as colony appearances were concerned, these colonies were no different from any other colonies of the Park 8 strain, being typically Sr. All of the various-sized colonies appeared to be composed of the same type of organisms.

The cells from several of the typical diphtheria colonies were stained by methylene blue. The organisms were variable in size and shape. There were solid-staining blue spheres and some

elongated spheres.   Some of the elongated spheres had a light-staining band in the center so that they appeared like a diplococcus.   Some of these blue cocci contained red granules within them, in other cases the small red granule protruded from the surface.   In addition to these various spherically shaped cells there were a few short solid-staining rods and sometimes these short rods stained very lightly and contained a reddish granule at each end of the rod.   These organisms did not at all resemble diphtheria organisms.   One of the colonies showing these peculiarly shaped organisms was transferred to a glycerol agar slant. There was good growth after 48 hours and the organisms in a

First   **Second**   **Third**

generations on solid media.
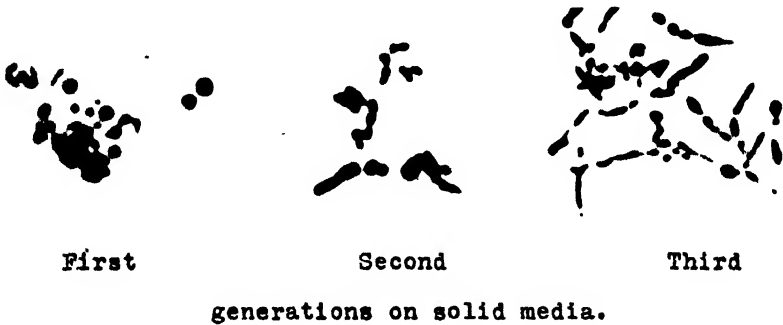
FIG. 3. C. DIPHTHERIAE, PARK 8 STRAIN
Aged 144 days in an ampoule at room temperature then transferred to glycerol infusion agar.   Methylene blue stain.   × 2650.

methylene-blue stained preparation still showed a very few of the spherically shaped cells, but for the most part the cells were of the rod form.   There were long thick rods, much larger than ordinary diphtheria bacilli, which contained both bars and granules and short rods which were thicker than the usual diphtheria rod.   The width of these short rods was about the same as the diameter of the cocci in the previous culture so that they appeared as if they were elongations of the spheres.   Third and fourth generations of this strain showed the organisms in practically their normal morphology, as shown in figures 2 and 3. The growth from a 24-hour-old blood agar slant culture killed a 300-gram guinea pig in less than 45 hours.   The organisms were

agglutinated by an agglutinating serum which had been prepared against the parent Park 8 strain.

At the same time that the glycerol agar plate was inoculated with three drops of the aged culture from the ampoule, three drops were also inoculated into glycerol infusion broth. The culture was propagated serially in glycerol infusion broth and showed the same transition in morphology as the culture did on glycerol agar. Only one difference was noted and that was that about twice as many generations were required in broth as on agar before the organisms returned to their usual morphology.

Thus, the granular-barred form described by Schultz was encountered. Upon other occasions it was observed in stained preparations of the diphtheria organisms, but it is not as common as Wesbrook's types. The fact that this form does occur warrants its inclusion in any table of morphological types of the organism. Since coccoid forms also have been observed by numerous workers, this form, too, should be included among the morphological forms.

<div align="center">DISCUSSION</div>

Inasmuch as smooth and rough colony variants exist for practically all species of microorganisms which have been studied at all extensively and since the terms *smooth* and *rough* are descriptive terms for such colony variants, have been found applicable to bacterial species in general and have priority to the terms *mitis* and *gravis* in reference to the diphtherial species, it is logical to attempt to explain the *mitis* and *gravis* variants in terms of the older and more generally used terminology. From the literature reviewed and the experimental work cited it appears that the *mitis* colonies are similar to the previously described smooth colonies for the diphtherial species, and the *gravis* colonies are similar to the larger, intermediate colony form. The group of colonies not classifiable as typically *mitis* and *gravis* appear to be nearer the rough phase than the larger intermediate or SR type colony.

A special medium containing potassium tellurite is not necessary for differentiation of the colony types. Unquestionably the

special tellurite medium may be very helpful when working with clinical material where one has present a mixture of microörganisms, some reducing the metal and the colonies thus becoming blackened, and others not reducing the metal and the colonies remaining uncolored. Anderson, Happold, McLeod and Thomson stated as one of the characteristics of the *mitis* type of colonies that the organisms are partially inhibited in their growth on the special potassium tellurite chocolate agar. This inhibition of growth of the *mitis* and of the S colony types has been observed—sometimes complete inhibition even when large inocula were used—so the question arises as to how many *mitis* or S strains might be missed when employing this medium to the exclusion of all others when searching for the diphtheria organism. In the study of 57 bacteriologically proven cases of diphtheria, Cooper, Peters and Wiseman (1939) reported the isolation of 4 strains of *C. diphtheriae* on Loeffler's medium which failed to appear on the potassium tellurite agar medium, Perry and Petran (1939) also report similar experiences. Their recommendation of the use of both media for the isolation of the diphtheria organism from clinical material is logical. A medium, in order to be perfectly satisfactory for the growth of the diphtheria organism and especially for isolating the organism from clinical material, should not exert an inhibitory action on any culture phase of the organism.

From the meager descriptions of the small colony forms in the literature it appears that the organisms are much smaller than those which are usually considered normal for the diphtherial species, and in the few instances in which the cultural reactions have been investigated, these organisms appear to require a somewhat longer period of time to bring about the various reactions which are usually considered characteristic of the diphtherial species. The observations on the strains of small colony variants described in this paper substantiate these earlier observations.

In 1935 the term "D," or dwarf, was employed (Morton, 1935b) to denote small colonies of the diphtheria bacillus because the organisms in these drawf colonies had not been shown to be filterable, had not gone through a stage in which their growth

was invisible, had not been obtained by any special bacterio-
logical technics and did not show a great variation from the
parent culture of normal, large size except for their relatively
small colony size and slowness in bringing about biochemical re-
actions.   It was considered best to discontinue the use of the
term "small" as sooner or later an abbreviation would be used,
and since the letter S already signified the smooth colony type.
The small colonies sometimes observed in other species of micro-
organisms have been designated as midget, minute or drawf
colonies.   The term midget and minute, while descriptive terms
for the colonies, are unsatisfactory as sooner or later they will
be abbreviated to the letter M which already denotes the mucoid
colony form.   Neither are these small colonies the C forms of
Kuhn and Sternberg (1931) as the morphology of the cells does
not resemble a coccus.   Moreover, Hadley (1933) announced
that his G colonies were similar to the C colonies of Kuhn and
Sternberg.   Since the size of the colonies and the physiological
activity of the organisms within the small colonies are dwarfed
in comparison to that which has been commonly recognized for
the species, the term D or dwarf was selected.   It signifies that
the size and activity of the organisms in this culture phase appear
small or slight in comparison to the colony sizes and activities of
those organisms commonly regarded as the normal size for the
diphtherial species.   Also the letter D was not being employed
for any other culture phase.   A later search of the literature
for evidence of similar small colony forms for other bacterial
species revealed that Eisenberg in 1914 also employed the term
dwarf to designate a small colony form of the typhoid bacillus.
This ability to exist in an extremely small colony form in addition
to the more common large colony forms, is not a characteristic
of the diphtherial species alone but is shared by about twenty
other bacterial species (Morton).

In 1931 Hadley, Delves and Klimek described at some length
the filterable form or G culture phase of the Shiga dysentery
bacillus and analogous forms of culture in 11 other species,
namely: *Escherichia coli, Eberthella typhosa, Salmonella paratyphi*
A and B, *Salmonella enteritidis, Salmonella cholerae-suis, Sal-*

monella typhimurium I, *Salmonella typhimurium* II (*Bacillus pestis-caviae*), *Corynebacterium diphtheriae* (Park 8 strain), *Lactobacillus acidophilus* and *Vibrio cholerae*. In the same year Kuhn and Stern-berg (1931) described several culture phases for bacteria, some of which were similar to the culture phases previously described by other workers. Hadley (1933) pointed out this similarity, stating that the C (Kokken formen) seemed definitely related to the G forms. In addition to producing C forms for *Escherichia coli*, *Eberthella typhosa*, *Salmonella typhimurium*, and *Corynebacterium diphtheriae*, for which Hadley, Delves and Klimek had described G forms, Kuhn and Sternberg produced C forms also from *Vibrio metchnikovi*, *Spirillum volutans*, *Bacillus anthracis*, *Bacillus pseudo-anthracis*, *Proteus vulgaris* and *Proteus* X-19, *Mycobac-terium tuberculosis*, *Salmonella suipestifer*, "Schweineseuche" and the dysentery bacillus of Shiga-Kruse.

Since 1931 small colonies and G colonies have been described for many bacterial species. Many of the small colonies referred to in the literature as G colonies are so named solely because of their small size; however, smallness of size is not the only criterion for the G culture phase. The small colonies of the diphtheria bacillus herein described as G colonies are so named because they fulfill the 5 postulates of Hadley (1931) for filterability and the G culture phase. This work is a confirmation of the experi-ments of Hadley and Richardson on the Park 8 strain which were described in the monograph by Hadley, Delves and Klimek. Haddow (1938) described G forms for *Salmonella paratyphi* B (Tidy) and reported the appearance of similar G colonies in cultures of *Eberthella typhosus* (Cole), *Salmonella paratyphi* A (Schottmüller) and a recently isolated strain of *Escherichia coli*. The G colonies for *Salmonella paratyphi* B (Tidy) fulfill the postulates of Hadley.

GENERAL CONCLUSIONS

A study of the diphtherial organism from many angles of approach reveals that its colonies on agar are characterized by at least four distinct, stable colony types and are not necessarily "small, grayish, granular, almost transparent, lace-like, margin

irregular" as described in Bergey's Manual for Determinative Bacteriology. As in other bacterial species, these colony types have been described as smooth (S), intermediate (SR), rough (R) and dwarf (D), according to their appearance on plain infusion agar.

Organisms from these four main colony types are true diphtheria bacilli, as judged by their tinctorial, cultural, and serological reactions and by their pathogenicity for guinea pigs.

With these differences in colonial appearances on plain agar can be associated variations in the manner of growth in liquid media, in stability in saline solutions of varying concentrations of sodium chloride, differences in appearance on potassium tellurite agar and certain quantitative variations in such properties as fermentation, toxigenicity, hemolysis, resistance to aging, etc.

There is order in the variation process within the diphtherial species. It is possible to predict, with a reasonable degree of certainty, the cultural reactions and the morphology of the organisms when a strain passes from one colony form to another. There is less certainty, however, which colony form will be the first to appear when a strain is undergoing forced dissociation. In contrast to some bacterial species, the variations in the characteristics of the organisms from the various colony forms are quantitative rather than qualitative.

In addition to the four stable colony forms of the diphtheria organism mentioned above, another phase, namely the G, was encountered. This G culture phase differs from all the other colony variants in that it is filterable, usually passes through a period of invisible growth, and when G colonies are obtained, by special culturing technics, the organisms differ from the more common colony forms biochemically, serologically and in pathogenicity. The characteristics of the G colonies of the diphtheria organism in relation to the larger and more common colony forms are not at all unlike the characteristics of the G colonies in relation to the larger colony forms of other species for which the G culture phase has been produced.

Unlike most other bacterial species the morphology of the diphtherial organism is very alterable without there being a

concomitant change in colony form. Because of its practical importance in diagnosis this aspect has been investigated quite extensively. The morphology of the cells differs with the physiological youth and age of the culture, the pH and composition of the medium, the manner in which the medium was sterilized, oxygen tension, symbiotic relationship with other organisms, and in different sections of the same colony.

Many discrepancies within the species reported by earlier workers can be explained by the quite normal variations of the diphtherial organism. These variations within the species are usually associated with the S, R, and D colony variation.

I am very grateful to Dr. Philip Hadley for his guidance and assistance in the earlier dissociative studies and to Dr. Stuart Mudd for many helpful suggestions.

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PLATE 1

THE FOUR MAIN COLONY TYPES OF THE DIPHTHERIA BACILLUS

FIG. 1. (upper left). *S*, smooth colony, *C. diphtheriae*, Park 8 strain, grown 48 hours on infusion agar at 37°C. Reflected light. ×12.

FIG. 2. (upper right). *SR*, intermediate colony, *C. diphtheriae*, Park 8 strain, grown 48 hours on infusion agar at 37°C. Reflected light. ×12. These two colonies *S* and *SR*, were growing side by side on the same infusion agar plate.

FIG. 3. (lower left). *R*, rough colony, *C. diphtheriae*, II strain, grown 48 hours on infusion agar at 37°C. Transmitted light. ×18.8.

FIG. 4. (lower right). *D*, dwarf colony, *C. diphtheriae*, Park 8 strain, grown 48 hours on infusion agar at 37°C. Transmitted light. ×18.8.
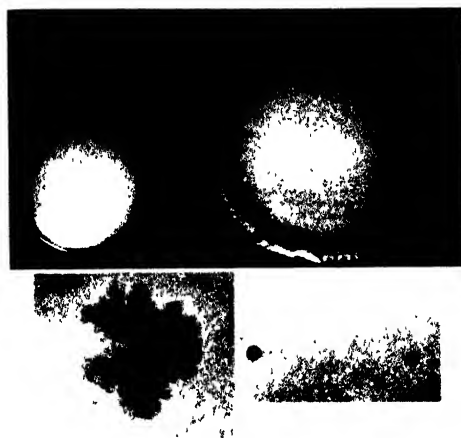
(Harry E Morton, Corynebacterium Diphtheriae)

THE BACTERICIDAL POWER OF SULFANILAMIDE UNDER ANAEROBIC CONDITIONS[1]

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As a result of the studies of Locke, Main, and Mellon (1938), Fox, German, and Janeway (1939), and others, and the chemical experiments of Shaffer (1939), all of whom have advocated the importance of the presence of oxygen and the formation of hydrogen peroxide as essential to the action of sulfanilamide, interest has developed as to whether sulfanilamide can exert its action on organisms *in vitro* under biologically anaerobic conditions.

Bliss and Long (1939) have reported that they were able to demonstrate bacteriostasis by sulfanilamide under anaerobic conditions, but did observe less effect at low oxygen tension. Broh-Kahn (1939) has likewise studied this question and substantiated the results of Bliss and Long. Using *Escherichia coli* as the test organism, this worker has elicited the following facts. When the organism is grown in nutrient extract broth it develops much better aerobically than anaerobically, and there is marked bacteriostasis with sulfanilamide under aerobic but not under anaerobic conditions. This was explained by stating that the drug affects (of the two types of nutrition) only the aerobic mechanism of nutrition while the anaerobic mechanism is unaffected. In evidence of this fact the degree of growth aerobically in the presence of the drug was claimed to be the same as anaerobically without the drug. Further evidence for this point of view was adduced by the observation that in the presence of glucose no effect of the drug was noted

either aerobically or anaerobically. In this case the glucose was assumed to supply a pathway of nutrition unaffected by the drug. However, when the organism was grown in a synthetic medium quite different results were obtained. *E. coli* grows well aerobically on the salt mixture of Quastel, Stephenson, and Whetham (1925) when one per cent lactate is added. In order to obtain anaerobic growth, however, it is necessary to add one per cent nitrate. It was found that sulfanilamide had no effect on this organism growing aerobically with or without nitrate, but that anaerobically there was marked bacteriostasis. The author explained this by assuming that sulfanilamide prevents the oxidation of lactate by nitrate and so exerts its effect. It was claimed by this worker that very rigid anaerobic conditions were attained, and that there were few molecules of oxygen present in the medium. Fox (1940) has criticized the findings of Broh-Kahn, claiming that reducing conditions were not established in the lactate-nitrate medium under anaerobic conditions as could he demonstrated by the fact that there was no reduction of methylene blue.

A bacteriostatic effect is by no means as clear cut as a bactericidal one, and it was therefore thought of interest, in the light of the findings of White and Parker (1938) that sulfanilamide is bactericidal at 40°C., to study the action at this temperature under anaerobic conditions.

The technique of White and Parker was followed mainly, with the exception that tryptose broth with one-tenth per cent glucose was used as the medium. 0.4 ml. of a 1:1,000 dilution of a 5-hour culture of the organism was seeded into 9 ml. of tryptose broth and thoroughly mixed. 4.0 ml. of the mixture was placed into each of two 50 ml. Erlenmeyer flasks (this produced a very thin layer of liquid, thus allowing more rapid equilibration with the atmosphere). To one flask was added 0.2 ml. of 0.44 per cent solution of sulfanilamide in distilled water, and to the control 0.2 ml. of sterile distilled water. The flasks were then incubated at 40°C. for 48 hours, at which time subcultures were

made and the viable bacterial population determined. Similar results were obtained when the cultures were incubated as tall narrow columns in tubes without shaking.

The anaerobiosis was produced in a jar in which phosphorus was ignited under sealed conditions (Varney, 1926). At all times, unburned phosphorus was present in order to react with any residual oxygen present. This method of producing anaerobiosis has been found satisfactory for growing the strictest anaerobes. The sulfanilamide solution was made up in water, double-distilled from glass, and prepared fresh every few days. The media were heated twenty minutes in flowing steam at 100°C. before inoculation in order to destroy any peroxides present. To further aid in the establishment of reducing conditions, 30 mgm. per cent of crystalline ascorbic acid was added to both the sulfanilamide and control flasks. A streptococcus, strain C203, was adapted to grow at 40°C. and was used for all the experiments involving streptococci.

The technique with *E. coli* was the same. Two strains of *E. coli*, "P.C." and "K3B", both of which grew well on the Quastel medium, were used in these experiments.

RESULTS WITH STREPTOCOCCUS

It was found that at 40°C., 20 mgm. per cent sulfanilamide was able to sterilize small inocula within 48 hours, but not in 24 hours, either aerobically or anaerobically, with or without the presence of ascorbic acid. A typical experiment is given in table 1.

Under these conditions sulfanilamide was not found to have any demonstrable action at 37°C. Suspecting that a possible explanation of this marked difference might be that at 37° the organism grew so rapidly that it overwhelmed the drug, we attempted to slow down this growth by diluting the medium with saline. No action of the drug could be demonstrated in this way. As the medium was diluted with saline till growth was less and less, there was exactly the same amount of growth in the flask containing drug as in the control, down to the point of cessation of growth. Similarly, the addition of 1:10,000

FeCl₃ to the medium in the hope of "potentiating" the drug had no effect. The reason for the striking temperature difference is at present obscure.

EXPERIMENTS WITH E. COLI

The results with *E. coli* are in some respects the same, and in some different from those with the streptococcus. Contrary to the results of Broh-Kahn, no action of the drug on *E. coli* grown in nutrient extract broth could be discerned. Since the possibility existed that this might be due to the presence of a

TABLE 1

Action of sulfanilamide on streptococcus C203 at 40°C. under aerobic and anaerobic conditions

	AEROBIC				ANAEROBIC			
	Ascorbic		Control		Ascorbic		Control	
	Sulf.	Cont.	Sulf.	Cont.	Sulf.	Cont.	Sulf.	Cont.
Gross appearance. . . .	Clear	Turbid	Clear	Turbid	Clear	Turbid	Clear	Turbid
Subcult. to broth.......	−	+	−	+	−	+	−	+
Subcult. to blood plate.	−	+	−	+	−	+	−	+
Bact. count per ml.*....	−	5.1×10^7	−	3.1×10^7	−	8.2×10^7	−	4.7×10^7

* Initial concentration of bacteria was 450 per ml.

different kind of peptone, we repeated the experiment using the tryptose-phosphate-glucose broth previously used for our streptococcus, nevertheless, no action was demonstrable.

With the Quastel synthetic medium, however, results entirely similar to those obtained with the streptococcus were demonstrated. The drug was able to sterilize, or at least very markedly inhibit the growth of small inocula (less than 5,000 per ml.) of *E. coli* under either anaerobic or aerobic conditions. Again, our results were somewhat different from those of Broh-Kahn in that we were able to get bactericidal action in the presence of glucose as well as the lactate-nitrate medium. The results with

this organism differed from those with the streptococcus in that there was marked action at 37°C., although there was no sterilization such as usually occurred at 40°C.

DISCUSSION

The question can of course be raised as to whether we have accomplished complete *chemical anaerobiosis*. The answer is probably, no. However, we have been able to obtain complete *biological* anaerobiosis in the sense usually employed in bacteriology, since this method of producing anaerobic conditions, i.e., by burning phosphorus, is quite sufficient for bacteriological purposes. The piece of phosphorus usually burns out in a matter of a few minutes thus establishing anaerobic conditions fairly rapidly. The addition of ascorbic acid was resorted to since it has been shown to be possible to grow anaerobes aerobically by the addition of this agent. The protective effect of glucose noted by Broh-Kahn and explained as offering an alternative pathway of nutrition has not been observed in our experiments. The observation of Lockwood (1938) that peptone interferes with the action of sulfanilamide has not been observed in our experiments with streptococci, since the tryptose broth contains 2 per cent peptone. The failure of the drug to act against *E. coli* remains unexplained, though in this case peptone may be concerned.

SUMMARY

1. At 40°C., sulfanilamide in a concentration of twenty milligrams per cent, in tryptose-phosphate-glucose broth, is able to sterilize small inocula of streptococcus C203 in 48 hours either aerobically or anaerobically with or without the presence of ascorbic acid.

2. No action under these conditions was noted at 37°C., and attempts to aid the drug by slowing down the growth of the organism by diluting the medium with saline were negative. Likewise, attempts to potentiate the drug by ferric chloride were negative.

3. Sulfanilamide had no effect on two strains of *Escherichia*

coli when grown in nutrient or tryptose broth aerobically or anaerobically.

4. In synthetic medium containing either glucose or lactate-nitrate at 37° or 40°C. marked inhibition or sterilization of small inocula of two strains of *E. coli* was noted both aerobically or anaerobically in the presence of ascorbic acid.

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THE CURATIVE EFFECT OF CERTAIN GOLD COMPOUNDS ON EXPERIMENTAL, PROLIFERATIVE, CHRONIC ARTHRITIS IN MICE[1]

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The purpose of this communication is to report experiments which demonstrate (1) the curative effect of certain gold compounds on an experimental, proliferative, and progressive, chronic arthritis in mice, (2) the conditions under which these compounds exert their optimum effect, (3) the relation between chemical composition and solubility on one hand and toxicity and therapeutic effectiveness on the other, and (4) the absence of any apparent *in vitro* effect of a therapeutically active compound, or of the blood of mice treated with it, against the microbial agent which causes the arthritis.

The experimental disease which has lent itself to the investigation of these questions has already been described briefly (Sabin, 1939a), and our present understanding of it and of the etiological microbial agent is somewhat as follows. Many different stocks of mice have been found to be carriers of a number of biologically and immunologically different types of filtrable microörganisms of the pleuropneumonia group (Sabin, 1939b). These microörganisms have been found in normal mice chiefly in the mucosa of the nose and sinuses and on the conjunctiva, and, only occasionally and in small numbers, in the lungs and brain; but examination of thousands of mice has thus far failed to reveal any spontaneous arthritis. However, when some of these normal microbial inhabitants of mice are cultured *in vitro* and injected

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[1] Part of the work presented in this communication was carried out while one of us (A. B. S.) was on the staff of the Rockefeller Institute for Medical Research.

intravenously even into mice of the same stock, they give rise to a progressive, proliferative chronic arthritis. The studies carried out thus far by one of us (A. B. S.) indicate that the pleuropneumonia-like microörganisms of mice have distinct tissue affinities (chiefly for those of mesenchymal origin) and in the animal body multiply within susceptible cells, even though outside the body it is possible to cultivate them in the absence of living cells. Some strains have been found to attack more types of mesenchymal cells than others, and the one selected for the present study (a type B strain) has its chief affinity for the joints. After a single intravenous injection of this strain the blood and organs are as a rule sterile within 24 hours and remain so while the arthritis develops and positive cultures are obtained from the joint tissues. Following a single intravenous injection of 0.5 ml. of a properly growing 24- to 48-hour culture of this strain (72-hour cultures are not pathogenic) 90 to 100 per cent of mice develop a polyarthritis which is migratory at first and finally progressive and chronic, leading to ankylosis in many instances between 2 to 4 months after inoculation. The incidence of ankylosis was very high (over 70 per cent) with this strain early after isolation, and ankylosis, as well as the essentially chronic character of the arthritis, have diminished with cultures between the 50th and 75th passage.

A Giemsa-stained film of a 48-hour serum glucose broth culture, used for the production of the experimental arthritis, is shown in figure 1. The complex morphology of this microbial agent is one of its striking characteristics but it is necessary to point out that the minimal reproductive unit is an elementary-body-like structure which according to gradocol membrane measurements is of the same order of magnitude as vaccinia virus. The gross appearance of the arthritis is illustrated in figure 2. The anterior and posterior extremities are equally affected and symmetrical involvement is quite frequent. Fusiform swelling of the digits is very common. The knee-joints are also affected but their involvement is not readily discernible clinically until partial or complete ankylosis has set in. Pathological changes are found in the capsule, synovia, cartilage and subchondral
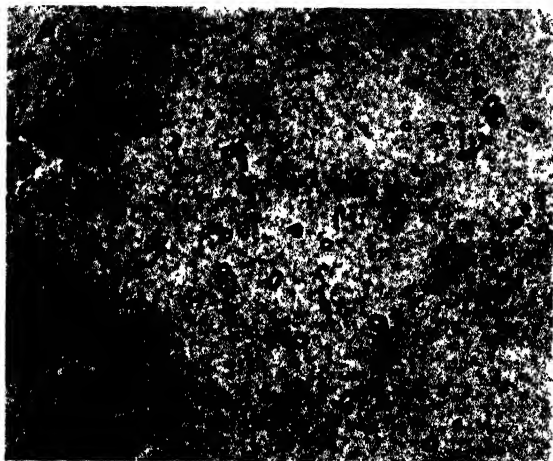
FIG. 1. FILM OF 48-HOUR CULTURE OF THE TYPE B MICROORGANISM USED FOR THE PRODUCTION OF EXPERIMENTAL CHRONIC ARTHRITIS
Giemsa, × 1,000
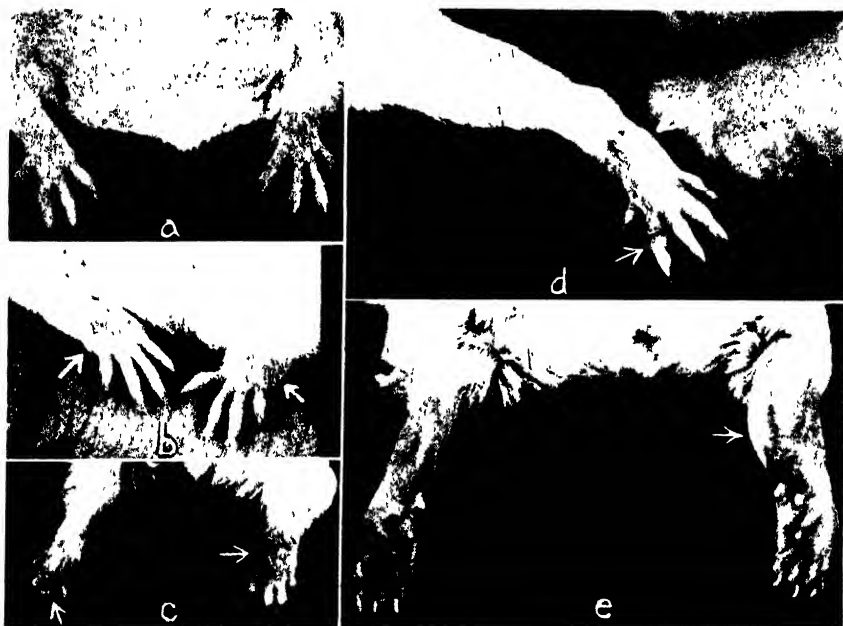
FIG. 2. GROSS APPEARANCE OF AFFECTED JOINTS

a, Anterior extremities of normal mouse. *b*, *c*, *d*, and *e*, extremities of mice with experimental arthritis of 1 to 2 weeks' duration; arrows point to involved joints. × 1.6.

bone-marrow (Sabin, 1940). The essentially proliferative character of the process is apparent in the synovia as early as the second day after swelling of the joint is discernible and becomes more marked subsequently, affecting the capsule, synovia and
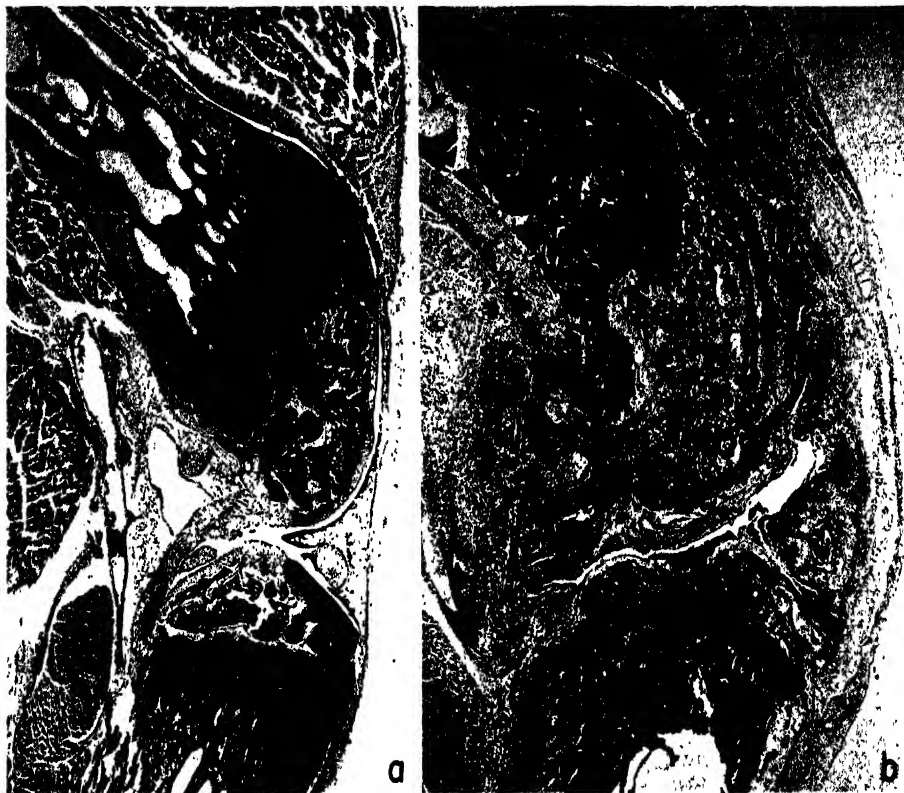
FIG. 3.   a, KNEE OF NORMAL MOUSE TO BE COMPARED WITH b, KNEE OF AFFECTED MOUSE 33 DAYS AFTER INJECTION OF CULTURE

Note (1) proliferation of tissue affecting synovia and capsule, articulating cartilage, and subchondral bone-marrow; (2) obliteration of free joint space by proliferated tissue; and (3) necrosis of articulating cartilage of femur and tibia. × 19.

perichondrium to such an extent that obliteration of the free joint space may result from an overgrowth of the proliferating tissue (see knee-joint in fig. 3). Actual destruction of cartilage was not seen until about the 4th week, and at 7 weeks one may find (as in the wrist in fig. 4) that the normal articulating cartilage

FIG. 4. a, WRIST OF NORMAL MOUSE FOR COMPARISON WITH b, WRIST OF MOUSE WITH EXPERIMENTAL ARTHRITIS 50 DAYS AFTER SINGLE INTRAVENOUS INJECTION OF CULTURE

Note (1) disappearance of most of the articulating cartilage and replacement by proliferating undifferentiated cells which may be immature chondroblasts or osteoblasts; (2) proliferative reaction in synovia and capsule. × 34.

has been replaced by undifferentiated cells which may be immature chondroblasts or osteoblasts. In a completely ankylosed joint, at about 5 months, one finds that considerable ossification has occurred in the distorted articulating surfaces which are joined by dense fibrous tissue.

While the gross appearance, the clinical course and the pathology of this experimental disease in mice are in many respects similar to rheumatoid arthritis in man, repeated attempts to obtain a similar microörganism from exudates and tissues of the human disease have been unsuccessful (Sabin, 1939b). Nevertheless, it appeared possible that certain problems relating to the therapy of human rheumatoid arthritis might be investigated with the help of this experimental disease in mice. All sorts of chemical compounds and biological agents have been tried empirically in rheumatoid arthritis, but because of the nature of the disease evaluation of results is so difficult that the editorial committee of the American Rheumatism Association was prompted to make the following comment in the Fifth Rheumatism Review (1939) (Hench *et al.*, 1939): "The curve of acceptance of most "new" treatments for arthritis that are destined to be discarded, rises rather rapidly, reaches its peak in about three to five years, then falls as adverse reports begin to outnumber the optimistic ones." Since Forestier's paper in 1929 there have been numerous communications especially from France and England, and lately from American investigators reporting with varying degrees of enthusiasm on the benficial effects of various gold compounds in rheumatoid arthritis, and the Fifth Rheumatism Review referred to this subject as follows: "American physicians, aware of the supposed value of gold for arthritis but concerned about its toxicity, have been slow to use it, expecting it would die out or be made safer. It therefore seems significant that the curve of acceptance of chrysotherapy is still rising after 10 years of use." It was of interest, therefore, to determine first whether compounds which have been reported to have a beneficial effect on the human disease, would behave similarly with regard to the experimental mouse arthritis. Quite recently Collier (1939) working with a spontaneous rat "ar-

thritis," transmissible by periarticular injection of infected tissues, and Findlay, Mackenzie and MacCallum (1940), with a spontaneous rat arthritis caused by a pleuropneumonia-like microörganism and some other pyogenic pleuropneumonia-like microörganisms of the rat, demonstrated that a number of gold compounds administered at the time of infection and subsequently could prevent the development of "arthritis" in most of the treated animals. With the problems of human therapy as a basis, our own experiments were curative rather than prophylactic in that treatment was not begun until the arthritis was well developed.

METHOD OF INVESTIGATION AND EVALUATION OF RESULTS

Culture. In order to be able to produce arthritis in at least 90 per cent of mice, it is necessary to have a culture that is growing under optimum conditions. The culture medium is important, in that a final pH that is more acid than 7.6 to 7.8 or the use of commercially dried beef heart instead of the fresh tissue for the preparation of the infusion has yielded no growth or poor growth, which upon intravenous injection into mice produced no arthritis. The culture medium we have used successfully is made up as follows: The broth is a fresh beef-heart infusion, containing 1 per cent peptone, and has a pH of 7.8 to 8.0 after sterilization. Glucose is added to a concentration of 0.5 per cent and bovine serum or ascitic fluid, which has been freshly filtered through a single Seitz disc, to a concentration of 10 to 30 per cent.

In order to overcome the gradual modification which occurs with prolonged *in vitro* passage, no cultures were used beyond the 50th transfer. Cultures in the 37th and the 43rd transfer were dried from the frozen state according to Swift's technique for preserving bacteria (1937). Since growth from the dried specimens is slow and not optimum, two or three rapid passages were made prior to animal inoculation. An actively growing culture would also be stored in the refrigerator and transfers from it could be made as required during the course of about a month. The inoculum for a culture that is to be used for mouse inoculation is obtained from a tube in which growth appeared in 24 to

48 hours and the amount is 1 or 2 parts of actively growing culture in 50 parts of fresh medium.

Animals. Albino mice, 3 to 4 weeks of age, were used in all experiments. 0.5 ml. of the culture was injected intravenously.

Observations and records. The minimum number of mice injected at one time was 30, but the usual number in a single experiment varied from 50 to 120. Mice were caged in groups of 10; each one was numbered so that a record could be kept of each mouse over a period of months. The joints of the anterior and posterior extremities were observed at frequent intervals, and an "arthrogram" (a diagrammatic record of the affected joints) of the type shown in chart I was made. With a good culture, arthritis often appeared as early as the 3rd or 4th day.

Therapy. No compound was administered until about a week after the appearance of arthritis in the majority of mice, which by that time were 5 to 6 weeks old and weighed 18 to 22 grams. The single minimal lethal dose for approximately 20-gram mice was determined for most compounds before they were administered. Most compounds that could be injected intravenously were given by that route, although the effects of administration by the intramuscular and subcutaneous routes were also studied. In each experiment there was a group of at least 10 mice which were untreated and which served as controls for the particular culture that was injected, as well as groups of animals given therapeutically ineffective doses or compounds, so that mice in which a definite therapeutic effect was observed would stand out in contrast to the others.

THERAPEUTIC EFFECT OF VARIOUS GOLD COMPOUNDS

A preliminary experiment, in which a group of 10 mice was given 2 mgm. of sodium aurothiomalate (myochrysine) intravenously every other day one week after the onset of arthritis clearly demonstrated the remarkable therapeutic effect of this compound. Within 4 days there was a distinct diminution in the swelling of all the affected joints and by the end of the first week the arthritis had disappeared completely in 80 per cent of the mice. With this observation at hand, a systematic study

was undertaken to determine the influence of dosage, route of administration, effect of optimum doses at various stages of the disease, and the relation between the chemical composition of various gold compounds and their therapeutic and toxic effects. The compounds used in the treatment of human rheumatoid arthritis have been inorganic, organic and colloidal. As an example of an inorganic compound the commonly used double thiosulfate of gold and sodium (sanocrysin) was selected; myochrysine, triphal, and solganal were the soluble organic compounds, and calaurol or myoral (calcium aurothioglycolate) was used as an example of an insoluble organic compound suspended in oil; two colloidal preparations, aurol-sulfide and 1 per cent metallic colloidal gold, were tested because they had been used in human beings in an attempt to avoid the toxic reactions which occur with the soluble compounds.[2] Because all the organic compounds contain sulfur, which is necessary to bind the gold to the organic radical, and because sanocrysin also contains sulfur, although in a different linkage, it was desirable to test gold chloride to determine whether or not gold itself could exert a therapeutic effect. The amount of compound that could bring about the complete disappearance of arthritis in 50 per cent or more of mice within the two-month period of observation was taken as the minimal therapeutic dose for comparative purposes.

Effect of sodium aurothiomalate (myochrysine): (a) *Influence of dosage*. To determine the minimal therapeutic dose, as well as to observe the relation between various doses of myochrysine and the rate of complete disappearance of arthritis, different groups of mice were given 2, 1, 0.5, 0.25, 0.1, and 0.02 mgm. of the compound intravenously approximately 1 week after the first appearance of arthritis. Each dose was repeated every 48 hours until the arthritis disappeared completely in at least 50 per cent of the mice or until 10 doses had been administered. While there is at

Effect of Sodium Aurothiomalate (Myochrysine) on Experimental Proliferative Arthritis

CHART I

EACH SQUARE REPRESENTS A MOUSE, AND THE THICKNESS OF THE SYMBOLS INDICATES THE SEVERITY OF ARTHRITIS
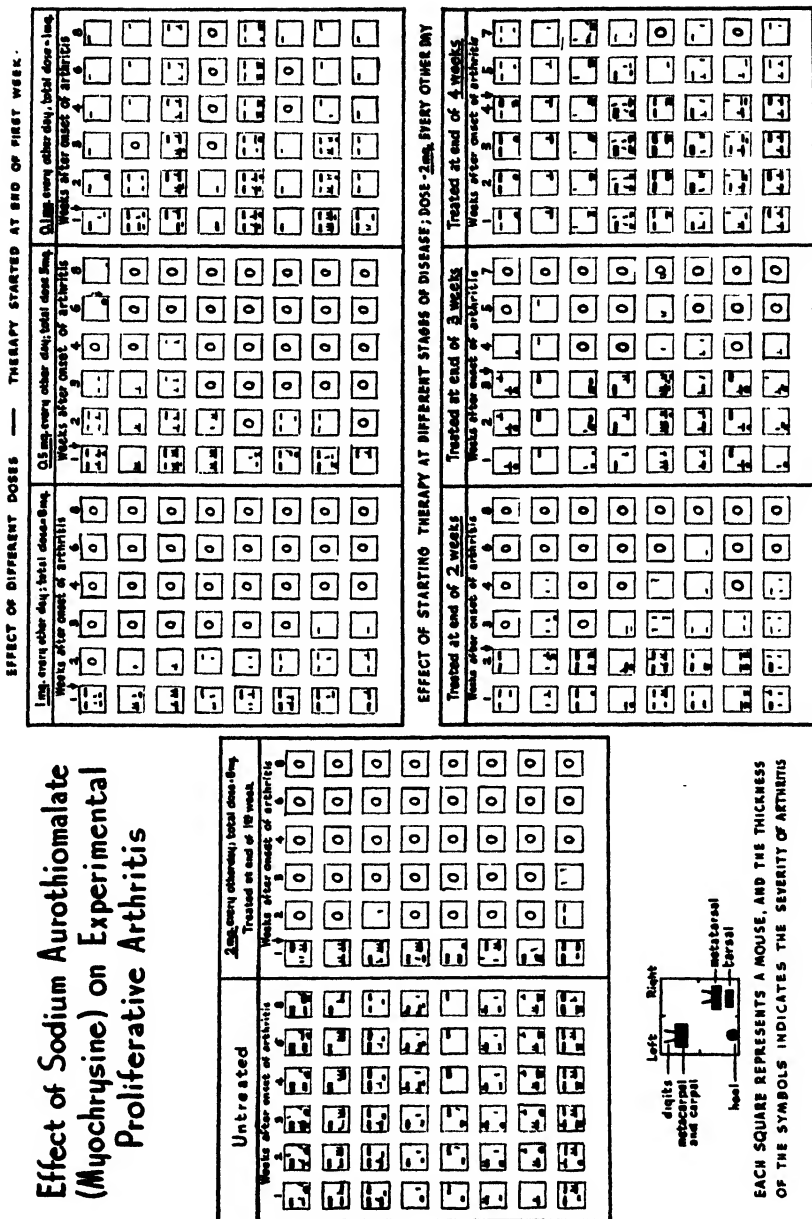
first a diminution in the severity of involvement, both as regards degree of swelling and number of joints affected in each mouse, it appeared desirable to use complete disappearance of the arthritis in an animal as the end-point for quantitative estimation. Arthrograms of groups of 8 mice, which were either untreated or had received varying amounts of myochrysine, are presented in chart I, which is intended to show the qualitative changes which occur in individual mice over a period of 8 weeks, while chart II presents the quantitative aspects of the same experiment.

RELATION BETWEEN DOSAGE OF SODIUM AUROTHIOMALATE (MYOCHRYSINE) & RATE OF COMPLETE DISAPPEARANCE OF ARTHRITIS
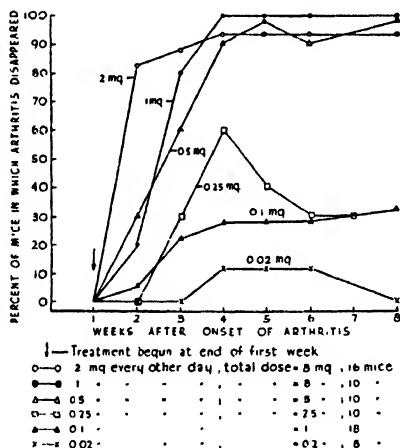
CHART II

The best result was obtained with the 2 mgm. dose since the arthritis disappeared in 82 per cent of the mice within the first week of treatment, in 88 per cent by the end of the 2nd, and in 94 per cent at the end of the 3rd week. While ultimately there was a clearing in all mice, there was a recurrence of the arthritis in a single digit of one animal during the 4th week, so that the final record remained at 94 per cent. With the 1 mgm. dose it was not until the end of the 2nd week that the arthritis had completely disappeared in 80 per cent of the mice, although at the

end of the first week there was already a halt to the further progress of the disease and a definite diminution in the severity and number of involved joints; 3 weeks after the beginning of therapy (or 4 weeks after the onset) all mice were free of arthritis. With the 0.5 mgm. dose the rate of complete disappearance of arthritis was still slower, although by the 4th week all had cleared; but there was a recurrence in a single mouse which disappeared, however, after further therapy (see chart I). The test with the 0.25 mgm. dose was not carried out until it was found that both the 0.02 and 0.1 mgm. amounts were definitely below the requirements of the minimal therapeutic dose. While the rate of disappearance of arthritis was slow, the 0.25 mgm. dose was regarded as the minimal therapeutic dose since arthritis completely disappeared in 60 per cent of the mice. It should be noted, however, that after cessation of treatment the incidence of recurrence was high, and it is open to question whether such a dose should be considered as the minimal therapeutic amount. The mice treated with 0.02 mgm. progressed as did the untreated animals, while among those treated with 0.1 mgm. there were quite a number in which the arthritis was much less severe or had disappeared altogether and then reappeared after a period of several weeks.

It is clear from these tests that not only the rate of disappearance of arthritis but also the percentage of animals in which it disappears is determined by the amount of myochrysine that is administered, and that on the borderline of the effective dose there is a high incidence of recurrence after the cessation of treatment. The mice which received individual doses higher than 0.25 mgm. and were free of arthritis at 6 to 8 weeks were still free when last observed at 6 months. That a single dose, provided it is large enough, is capable of exerting a curative effect was evident in a test in which the arthritis completely disappeared in 5 of 7 mice which were given 2 mgm. of myochrysine intravenously in one injection, and it is interesting to note that it took 2 to 3 weeks for the effect to occur. When it is recalled that repetition of the 2 mgm. dose every 48 hours brings about a similar therapeutic effect within 5 to 7 days after the

beginning of therapy, it becomes clear how dosage affects the outcome. Furthermore, when the same total dose (8 mgm.) was given over a period of 2 weeks in 1 mgm. amounts on alternate days it took twice as long for an equivalent therapeutic effect to occur as when administered over a period of 1 week in 2 mgm. amounts.

(b) *Therapeutic effect in relation to stage of disease when treatment is begun.* The question considered in the next series of tests was whether a compound like myochrysine, which exerted an excellent therapeutic effect when administered one week after

RELATION BETWEEN TIME OF STARTING THERAPY
& RATE OF COMPLETE DISAPPEARANCE OF ARTHRITIS
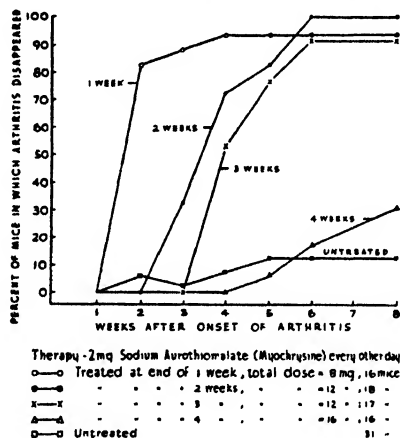
CHART III

the onset of arthritis, could produce similar effects at all stages of the disease, or whether there was some limiting time beyond which it would become inactive. Charts I and III show the effect of beginning therapy 2, 3, and 4 weeks after the onset of arthritis. Two milligrams of myochrysine was given intravenously every other day for a period of 2 to 3 weeks. It may be seen that when therapy was delayed as long as 3 weeks, a good result was still attainable, although the rate of complete disappearance of arthritis was considerably slower, as compared with the group which was treated one week after onset. When

treatment was delayed as long as 4 weeks, it was evident that in the majority of mice some joints, if not all, had progressed to a point where this therapy was no longer effective. The same was found to be true in a small group of mice which were treated similarly 10 weeks after the onset of arthritis, i.e. while the arthritis disappeared in some joints, there were others in the same mouse in which the condition was unaffected. Mice with ankylosis, 5 and 6 months after onset, were not visibly affected by treatment, although again there was possibly a good effect on an occasional joint which was not ankylosed. The time when this therapy can no longer bring about a complete disappearance of arthritis coincides with the stage when distinct cartilage destruction is first observed, and it would appear that a return to normal can be expected only in joints with cartilage that is not too markedly involved by the disease process.

Relation between route of inoculation and effect. Several groups of mice were given myochrysine intramuscularly or subcutaneously one week after onset of arthritis. With the 2 mgm. dose either intramuscularly (17 mice) or subcutaneously (8 mice) the therapeutic result was both as good and as rapid as after intravenous administration of the drug. By the intramuscular route, 0.5 mgm. was effective and the 0.1 mgm. dose was not, indicating that the intramuscular and intravenous minimal therpeutic doses were probably in the same range.
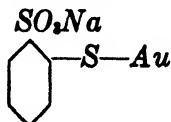
$$SO_2Na$$

Effect of solganal—$NH \cdot CH_2SO_2Na$. Solganal is an aurothio derivative of the formaldehyde bisulfite addition product of sodium sulfanilate and is not to be confused with solganol B which is aurothioglucose. Only enough solganal was available to treat eight mice which were given 2.75 mgm. (i.e. 1 mgm. gold) on alternate days for 4 doses. The response was as rapid and complete as with the equivalent dosage of myochrysine, the arthritis having disappeared in all the mice without recurrence for at least 6 months.

Effect of triphal (sodium aurothiobenzimid-azole-carboxylic acid). This compound is still under investigation but in view of the fact that it was found to be considerably more toxic by the intravenous route than myochrysine, it should be noted that the available results indicate that, while it can also exert a curative effect, the minimal therapeutic dose does not appear to be smaller than that of myochrysine.

Effect of sanocrysin (double thiosulfate of gold and sodium). The inorganic compound, sanocrysin, as will be shown later, is

EFFECT OF DIFFERENT DOSES OF DOUBLE
THIOSULPHATE OF GOLD AND
SODIUM (SANOCRYSIN)

CHART IV

definitely more toxic than myochrysine, and its therapeutic effect had to be tested in smaller doses. The drug was injected intravenously one week after the onset of arthritis and the results shown in chart IV indicate that it can be quite as effective as myochrysine. While the minimal therapeutic dose in milligrams of sanocrysin (37 per cent gold) is greater than that of myochrysine (50 per cent gold), it is not improbable that in terms of actual gold, the minimal effective amount may be the same for both compounds.

Effect of calcium aurothioglycolate (calaurol, myoral). Calaurol,

an insoluble, organic gold compound, containing 64 per cent gold, was available as a 10 per cent suspension in oil of sweet almonds and was injected intramuscularly in 10 mgm. doses which were repeated every other day until 80 mgm. were administered. The drug was given to 9 mice one week after the onset of arthritis. For two weeks after the beginning of therapy there was no noticeable effect, but during the 3rd week the arthritis began to diminish and disappear so that by the end of that week it had completely disappeared in 7 of the 9 mice; in the course of the next two weeks the slight residual involvement of two joints of the remaining two mice also cleared up, and there was no recurrence in any of the animals. The therapeutic effect exerted by this "insoluble" organic gold compound indicates that it is probably slightly soluble in the body and is slowly absorbed from the site of inoculation. The fact that the largest amount of it (100 mgm. in 1 ml. of oil) that was given as a single dose to 20-gram mice was not toxic, suggests that further, more detailed, investigation of this type of compound is definitely indicated.

Effect of colloidal gold. Seventeen mice were treated with Aurol-Sulfide, which is described as a 0.5 per cent aqueous colloidal gold sulfide preparation containing 87 per cent gold and 13 per cent sulfur. One-half milliliter, containing 2.5 mgm. of the compound, which is the largest amount of the preparation that could be given in a single intravenous injection, to 20-gram mice, gave rise to severe tremors and prostration immediately after administration but all animals recovered and remained well. The first group of 9 mice received 1 mgm. every other day until 8 mgm. were administered; the first six doses were given intravenously but the remaining ones had to be injected intramuscularly because the veins had become occluded. Another group of 8 mice received a series of 9 doses of 1.5 mgm. each in the same manner. Of the 17 mice, 5 died during the first three weeks of treatment, and in the remaining 12 there was no therapeutic effect whatever. Although the affected extremities became colored in a manner that suggested a concentrated deposition of the colloid in the inflamed joints, the arthritis became progres-

sively more severe in the treated mice, and there was no difference between them and the untreated animals except that none of the untreated ones died.

Another colloidal preparation which was described as a 1 per cent suspension of metallic gold protected against precipitation by a small amount of gelatin and other "emulsoid colloids," was given to 9 mice, one week after the onset of arthritis. Five milligrams of colloidal gold were injected intramuscularly every 48 hours, but the injections were stopped after 20 mgm. had been administered because 3 of the 9 mice had died. The 6 surviving ones showed deposition of the colloid in the affected joints, but there was again no therapeutic effect of any kind.

Is sulfur a necessary part of a therapeutically effective gold compound? Effect of gold chloride. The following tests were carried out chiefly to determine whether a gold compound without any sulfur in it could exert a therapeutic effect on the experimental mouse arthritis. Gold chloride, $AuCl_3 \cdot HCl \cdot 3H_2O$, is highly toxic on intravenous administration; while the single minimal lethal dose (killing 50 per cent or more of 20-gram mice) is slightly more than 0.5 mgm., 0.1 mgm. was selected as the dose for treatment, because even 0.2 mgm. was found to kill a varying percentage of mice. Eight or nine doses, over a period of 2 to 3 weeks, were administered to 18 mice, intravenously at first and then intramuscularly as the veins became occluded after 3 or 4 injections. None of these animals died and they behaved in general like the ones treated with 0.1 mgm. of myochrysine, except that 5 weeks after the beginning of therapy the arthritis had completely disappeared in 50 per cent of the gold chloride group. Whether or not larger amounts of gold chloride could exert a greater and more rapid therapeutic effect could be determined only by the administration of doses which would kill a certain number of the mice. It was found, for example, that mice would survive a single subcutaneous injection of 15 mgm. of gold chloride although there was extensive local necrosis; while repeated injections of 2 mgm. amounts every other day killed about 50 per cent of the mice in one to 2 weeks. These necrotizing and toxic properties of gold chloride on subcutaneous in-

jection were not appreciably modified by adjusting the solution to a pH of 7.0 with tenth-normal sodium hydroxide, even though this neutral preparation was less toxic on intravenous injection. Twenty mice received 2 mgm. amounts of the acid or neutral solution of gold chloride subcutaneously one week after the onset of arthritis; 11 mice had a total dose of 7 mgm. of the acid gold chloride and 9 mice a total dose of 14 mgm. of the neutral solution. Of the 20 mice, 7 died within less than a week, before any evidence of a therapeutic effect could be seen; in the remaining 13, the arthritis disappeared completely between the first and second

RELATION BETWEEN SOLUBILITY OF GOLD COM-
POUNDS USED & THEIR THERAPEUTIC EFFECT
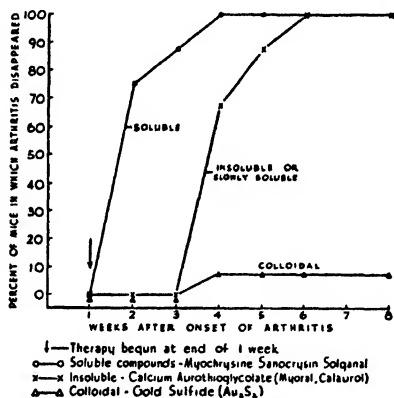
CHART V

weeks after the beginning of therapy; 4 additional mice died during that period, but the 9 survivors remained free of arthritis.

Thus, while gold chloride is too toxic for practical and even experimental therapeutic purposes, it is nevertheless clear that a gold compound without sulfur in the molecule is also capable of exerting a curative effect on the experimental mouse arthritis.

Solubility and therapeutic effect. The foregoing experiments suggest that the therapeutic effect exhibited by the gold compounds studied is dependent upon the availability of a certain concentration of chemically reactive gold, probably in an ionic state. This relationship is illustrated graphically in chart V.

The greatest and most rapid therapeutic effect was obtained with the largest doses of the soluble, crystalloid, inorganic or organic compounds; with an "insoluble" or slowly soluble compound from which chemically active gold is probably slowly liberated, the therapeutic effect is markedly delayed but nevertheless striking. In the colloidal state, however, gold has no therapeutic properties.

THE INEFFECTIVENESS OF SOME OTHER CHEMOTHERAPEUTIC AGENTS

Sodium salicylate. Sodium salicylate had no effect when administered subcutaneously either before or after the appearance of arthritis. Twenty mice were used in this test, each one receiving a total dose of 40 mgm. over a period of 8 days.

Bismuth subsalicylate. The intramuscular M.L.D. of this compound for 20-gram mice was between 40 and 60 mgm. Each of 9 mice was given 2.6 mgm. on alternate days for 8 doses (total—20.8 mgm.) and 10 mice received 13 mgm. each, every other day for 10 doses (total—130 mgm.) one week after the onset of arthritis; there was no evidence of a curative effect in either group.

Sodium sulfapyridine. The single intravenous M.L.D. of this compound for 20-gram mice was 10 to 12 mgm. Each of 10 mice received 5 mgm. intravenously every other day until a total dose of 35.0 mgm. had been administered. Treatment was started one week after the onset of arthritis and there was no evidence of any therapeutic effect.

Neoarsphenamine. The intravenous M.L.D. for 20-gram mice was 8 mgm. Administered intravenously every other day in 5 mgm. amounts to each of 10 mice for 8 doses, the drug exerted no appreciable effect.

SUMMARY OF CHEMOTHERAPEUTIC EXPERIMENTS

The essential results of all the preceding experiments, summarized in table 1, show that arthritis disappeared completely in 96 per cent of 171 mice which were treated with suitable gold compounds in adequate dosage and at the proper time, but in

only 7 per cent of 70 mice which were untreated and in only 5 per cent of 77 mice which received colloidal gold, sodium salicylate, bismuth subsalicylate, neoarsphenamine or sodium sulfapyridine in simultaneous tests and under identical conditions.

TOXICITY OF THERAPEUTICALLY EFFECTIVE COMPOUNDS

If the same factor or factors were responsible both for the therapeutic effectiveness and the toxicity of a compound one would expect the chemotherapeutic indexes of various active preparations to be of the same order of magnitude, and there would be little or no basis for attempting to find or synthesize

TABLE 1

Results of chemotherapeutic experiments

THERAPY	NUMBER OF MICE	PERCENTAGE OF MICE IN WHICH ARTHRITIS COMPLETELY DISAPPEARED WITHIN 8 WEEKS
Suitable gold compounds in adequate dosage at proper time	171	96 per cent
Suitable gold compounds, but in inadequate dosage or too late in disease	83	0 to 33 per cent in different groups
Colloidal gold, sodium salicylate, bismuth, subsalicylate, sodium sulfapyridine, neoarsphenamine	77	5 per cent
Untreated	70	7 per cent

additional gold compounds with a still greater margin of safety between the curative and toxic dosages.   The toxicity of a number of gold compounds of different chemical composition was, therefore, investigated in some detail and the results showed that there can be a wide range in the toxicity as well as in the chemotherapeutic index $\left(\dfrac{\text{minimal lethal dose}}{\text{minimal therapeutic dose}}\right)$ of the different compounds.

The single minimal lethal dose (M.L.D.) was determined in at least two steps.   In a preliminary test, groups of two to four 20-gram mice were injected with a number of graded doses in

order to find the zone which separated death and survival. The test was then repeated with a minimum of 6 mice for each dose within that zone, and the amount that killed 50 per cent or more of 20-gram mice during an observation period of 2 to 3 weeks was considered as the single M.L.D. The results of such tests are shown in table 2. For the intravenous route the single M.L.D. of a number of therapeutically effective gold compounds was found to be approximately as follows:[3]

Gold chloride, acid ... .   0.75 mgm. or 0.37 mgm. gold
Gold chloride, neutral . . . . . .   2.0 mgm. or 1.0 mgm. gold
Double thiosulphate of gold and sodium
    (sanocrysin) .   . . .   .   .   1.0 mgm. or 0.37 mgm. gold
Sodium   auro-thio-benzimid-azole-car-
    boxylic acid (triphal)   .   . 2.0 mgm. or 0.88 mgm. gold
Sodium aurothiomalate (myochrysine)... 8.0 mgm. or 4.00 mgm. gold

It is thus clear that the amount of gold in an M.L.D. can vary over a wide range in both inorganic and organic compounds. Since gold is the constituent responsible for the toxicity of all these soluble compounds, it would appear that the nature of the rest of the molecule and the manner in which it is combined with or dissociated from it probably determine not only how much of a certain substance shall be lethal but also (because the toxic signs after intravenous injection are not the same with all compounds) the type of toxicity that is produced. Myochrysine, for example, invariably killed with signs suggestive of respiratory failure within a few seconds after the injection and the unaffected mice, with only two exceptions, remained well thereafter. With sanocrysin, on the other hand, death was always delayed; and while 20 times the M.L.D. gave rise to convulsions and death in 6 to 9 minutes, smaller amounts frequently produced no obvious signs for 2 to 3 days with a still longer delay before death occurred. The sick sanocrysin mice showed particularly severe

[3] Furthur experience with toxicity tests has convinced us that for an accurate determination of the MLD approximately 20 mice must be used for each dose in the critical zone. Using these larger numbers of mice the intravenous MLD was found to be 7.5 mgm. instead of 8 mgm. for myochrysine, and 3 mgm. instead of 2 mgm. for triphal.

nervous signs consisting of marked gross tremors and ataxic gait associated with ruffled fur.

The relative behavior of these compounds after intramuscular injection was also peculiar in several respects. Myochrysine, even with the largest doses, killed after a delay of one or more days; the sick mice showed a hunched back, rapid respiration, and while they were hypersensitive to stimuli they were abnormally quiet when undisturbed and had none of the nervous signs exhibited by the sanocrysin animals and, furthermore, many of the sick mice recovered. While there was considerable irregularity in individual behavior, so that the exact intramuscular M.L.D. for myochrysine could not be interpolated from the number of mice that were used, it is nevertheless obvious that more of the compound can be tolerated by the intramuscular than by the intravenous route. There was greater regularity in reaction after subcutaneous injection and it appears that myochrysine is only about one half as toxic subcutaneously as intravenously.[4] This disproportion in the M.L.D. by different routes was even more marked for some of the other compounds. Gold chloride injected subcutaneously could be tolerated in a dose that was at least 20 times the intravenous M.L.D.; the extensive necrosis and gangrene which developed in a few days suggested that it practically all combined with the tissues at the site of inoculation. Gold chloride adjusted to pH 7.0 still produced local necrosis. On the other hand another inorganic compound, sanocrysin, which can be injected intramuscularly without producing appreciable local reaction was much more toxic and the intramuscular M.L.D. was only twice as large as the intravenous M.L.D.[5] That a disproportionate difference in toxicity by different routes can also occur with an organic compound containing the Au-S linkage is evident from the tests with triphal, which was approximately 3 times more toxic by the intravenous

[4] Additional tests with larger numbers of mice indicated that the MLD of myochrysine is 15 mgm. by the intramuscular route and 18 mgm. by the subcutaneous route.

[5] Additional tests with larger numbers of mice indicated that the MLD of sanocrysin by the intramuscular route is 1.5 mgm., and, therefore, only one and one-half as large as the intravenous MLD.

than by the intramuscular route without evidence of necrosis or other obvious injury at the site of inoculation.

While further work is necessary to determine the exact minimal therapeutic dose for a larger number of gold compounds administered by various routes, it is already evident from a study of the data obtained with intravenously injected sanocrysin and myochrysine that the factors which determine the therapeutic and toxic properties of a compound are not identical; the chemotherapeutic index for sanocrysin was approximately 2 as compared with 30 for myochrysine. Although there are as yet insufficient data for calculating the chemotherapeutic index for an "insoluble," organic gold compound, the preliminary observations on calcium aurothioglycolate point in the same direction, in that it exerted a striking curative effect, although after considerable delay, while even the largest amount that could be administered in a single dose gave rise to no signs of toxicity.

Several tests (table 2) with mice weighing less than 20 grams indicated, as was to be expected, that the size of the M.L.D. depends on the weight of the mouse but it should be noted that the relationship is not strictly proportional. For example, 10-grams mice tolerated 4 mgm. of myochrysine intravenously, while all 20-gram mice, which were given 8 mgm., died. In other words one cannot interpolate the toxicity of these compounds for animals heavier or lighter than 20 grams because, in proportion to weight, the heavier ones can tolerate less and the lighter ones more. It would, therefore, be misleading to describe toxicity in milligrams of compound per gram or kilogram of mouse. That being the case for mice of different age and weight it is clear that one cannot transpose the mouse-toxicity data to other animals or man simply by taking into consideration the difference in weight.

The data presented thus far have dealt with single-dose toxicity and studies on possible differences in excretion and cumulative effect of different compounds are still to be made. But it is already apparent that with the soluble gold compounds the cumulative effect is probably of greater significance as regards therapy than toxicity. Thus, an amount of myochrysine equiv-

TABLE 2

Toxicity of various types of gold compounds

COMPOUND	ROUTE	APPROXIMATE WEIGHT OF MICE	DOSE	NUMBER OF MICE	RESULT TIME OF DEATH IN DAYS
		grams	*mgm.*		
			5.0	4	D, D, D, D
			1.0	11	D, D, D, D, D, D, D, D, D, 1, 0
			0.75	6	D, D, D, D, D, D
	Intra-ve-nous	20	0.5	2	D, D
			0.5	6	D, 0.25, 0.25, 0, 0, 0
			0.5	6	D, 0, 0, 0, 0, 0
			0.5	6	1, 0, 0, 0, 0, 0
Gold chloride (acid)			0.2	2	D, 0
$AuCl_3 \cdot HCl \cdot 3H_2O$			0.2	4	D, D, S, 0
(50% Gold)			0.2	6	0, 0, 0, 0, 0, 0
			0.2	6	0, 0, 0, 0, 0, 0
			0.1	25	All well
	Subcu-tane-ous	20	15.0	6	Local necrosis; all survived
			10.0	6	Local necrosis; 1 dead 12th day
			5.0	6	Local necrosis; all survived
Gold chloride (adjusted to pH 7.0 with N/10 NaOH)	Intra-ve-nous	20	5.0	4	D, D, D, D
			4.0	6	D, D, D, 0.02, 0.02, 0.02
			3.0	16	(0.02) x 11, 2, S, S, 0, 0
			2.0	12	D, (0.02) x 9, 1, 1
			1.0	10	All well
	Intra-ve-nous	20	20.0	2	0.004, 0.006
			10.0	3	0.17, 1, 1
			5.0	7	1, 1, 1, 1, 1, 1.5, S, 0
Double thiosulphate of gold and sodium (Sano-crysin)			4.0	4	1, 1, 1.5, 1.5
			3.0	4	1.5, 4, 5, S
			2.0	4	4, 5, S, S
$Na_2Au(S_2O_3)_2 \cdot 2H_2O$			1.5	6	2, 4, 4, 6, 6, 18
(37% Gold)			1.0	6	5, 5, 5, 7, S, 0
			0.8	6	All well
			0.5	3	All well
	Intra-mus-cu-lar	20	4.0	6	1, 2, 2, 2, 2, 5
			3.0	6	2, 2, 2, 2, 2, 3
			2.0	6	2, 5, 5, 5, 5, 7
			1.0	6	All well

D—died instantaneously; S—sick, but recovered; 0—appeared well. Additional toxicity tests with some of these compounds have yielded somewhat more accurate end-points, which are mentioned in footnotes to the text.

TABLE 2—*Concluded*

COMPOUND	ROUTE	APPROXIMATE WEIGHT OF MICE	DOSE	NUMBER OF MICE	RESULT TIME OF DEATH IN DAYS
		grams	*mgm.*		
	Intra-ve-nous	20	25.0	2	D, D
			10.0	8	D, D, D, D, D, D, D, D
			8.0	6	D, D, D, D, D, D
			7.0	6	D, 4, 0, 0, 0, 0
			6.0	6	5, 0, 0, 0, 0, 0
			5.0	3	All well
			4.0	6	All well
		10	10.0	3	D, D, D
			8.0	3	D, D, D
			6.0	3	D, D, D
Sodium aurothiomalate			4.0	3	0, 0, 0
(Myochrysine) $CH_2 \cdot COONa$	Intra-mus-cu-lar	20	50.0	4	0.25, 1, 1, 1
$\mid$			25.0	4	1, 1, 2, 5
$Au—S—CH \cdot COONa$			10.0	10	3, 5, 5, S, S, S, S, S, 0, 0
(50% Gold)			8.0	6	5, 6, 7, S, S, S
			6.0	6	2, 0, 0, 0, 0, 0
			4.0	6	All well
		14	20.0	3	4, 6, 0
		14	10.0	3	6, 6, S
		11	6.0	7	2, 5, 6, 7, S, S, S
		13	5.0	7	5, 5, 6, 6, 7, 8, S
	Subcu-tane-ous	20	*15.0*	6	5, 5, 7, S, S, S
			10.0	6	5, S, 0, 0, 0, 0
			8.0	10	S, S, 0, 0, 0, 0, 0, 0, 0, 0
	Intra-ve-nous	20	5.0	6	0.04, 0.04, 0.04, 2, 2, 0
			3.0	12	(0.02) x 3, (0.04) x 3, 4, S, S, 0, 0, 0
Sodium aurothio-benzi-mid-azole-carboxylic			*2.0*	6	1, 1, 4, S, 0, 0
acid (Triphal)			1.0	6	All well
(44% Gold)	Intra-mus-cu-lar	20	*10.0*	6	1, 1, 2, 7, S, S
			7.0	6	S, S, 0, 0, 0, 0
			5.0	4	All well
			3.0	4	All well
			1.0	4	All well
Calcium aurothioglycolate (Calaurol, Myoral)	Intra-mus-cu-lar	20	100.0	14	All well
			50.0	3	All well
$Au—S—CH_2 \cdot COO$			40.0	10	All well
$Au—S—CH_2 \cdot COO$ $\rangle Ca$			30.0	3	All well
(67% Gold)					

alent to one or two M.L.D. was perfectly tolerated when injected in divided doses on alternate days over a period of one or two weeks. An amount of sanocrysin equivalent to 2 M.L.D. administered in 3 divided doses over a period of 4 days was harmless, even though the lethal effects of a single M.L.D. are delayed for 5 to 7 days. It would appear, therefore, that a part of the injected compound is excreted and another part stored and that while the therapeutic effect is determined by the amount stored in certain tissues, toxicity is more a function of the quantity of free chemically-reactive gold that is available at any one time.

MODE OF ACTION—EFFECT OF MYOCHRYSINE IN VITRO

The first question to be investigated in a study of the mode of action of these therapeutically active gold compounds is whether or not they can exert any effect *in vitro* on the microbial agent which is the cause of the experimental mouse arthritis. Myochrysine was selected for this study because it had the best chemotherapeutic index among the group. The possible direct effect of myochrysine on the microörganism was studied in a number of different ways, but no direct action was demonstrable by any of the methods.

(a) *Effect of various concentrations of myochrysine incorporated into solid medium.* Myochrysine in final concentrations of 1:1,000, 1:2,000, 1:5,000, 1:10,000, and 1:100,000, i.e. in concentrations of 100 mgm. to 1 mgm. per cent, was incorporated into 30 per cent ascitic fluid agar medium, and 0.05 ml. of culture was spread on the solidified medium in Petri dishes. With the exception of the 1:1,000 plates, in which there was marked precipitation of the medium, colonies developed as well on the agar containing myochrysine, even in concentrations by far exceeding those which might possibly obtain in the living animal, as on the control medium (table 3).

(b) *Effect of myochrysine in fluid media.* Since this microorganism cannot grow in the absence of a certain concentration of protein, the inhibitory or microbicidal effect of myochrysine in fluid medium had to be studied in broth containing at least 10

per cent of serum (i.e. about 0.6 to 0.8 per cent protein).[6] In one test a series of tubes containing 4.9 ml. of broth with 0.5 per cent glucose and 30 per cent ascitic fluid were seeded with 0.1 ml. of culture and myochrysine added in various amounts to reach final concentrations of 1:500, 1:1,000, 1:2,500, and 1:5,000. Although there was heavy precipitation in the 1:500 and 1:1,000 tubes and some precipitation in the other tubes, good growth occurred in all without evidence of inhibition or delay; proof of growth was obtained by Giemsa-stained films and subculture on fluid and solid media. Plating out of the various mixtures after 3 hours' incubation gave no indication that there might have been an

TABLE 3

Effect of various concentrations of myochrysine on growth of the type B microörganism of the mouse pleuropneumonia group in vitro

CONCENTRATION OF MYOCHRYSINE		GROWTH	
Mgm. per cent	Final dilution	Solid medium	Fluid medium
200	1:500		+
100	1:1000	0	+
50	1:2000	+	
40	1:2500		+
20	1:5,000	+	+
10	1:10,000	+	
5	1:20,000	+	
1	1:100,000	+	

initial microbicidal effect with subsequent growth resulting from a few surviving microorganisms. In another series of tests it was found that myochrysine in concentrations of 1 mgm. and 0.1 mgm. per 5 ml. of 10 per cent bovine serum broth (i.e. 1:5,000 and 1:50,000) does not prevent growth even when the inoculum contains minimal numbers of microorganisms. (See table 4.) Results similar to those shown in table 4 were also obtained when different amounts of a 48-hour culture were used as the inocula.

[6] After centrifugation and suspension in phosphate buffer of pH 7.35, these microorganisms survive for only a few hours at 37°C., and there was no evidence that addition of myochrysine in final concentrations of 1:1000 to 1:100,000 to such a suspension accelerated death of the microorganisms.

(c) *Effect of blood of myochrysine-treated animals.* Since there was no evidence that myochrysine, *per se*, in concentrations which may be considered equivalent to or larger than those which exert a distinct curative effect in the animal, either killed or prevented growth of the microörganisms *in vitro*, it was necessary to determine whether the blood of myochrysine-treated mice could exert such an effect. Preliminary tests with heparinized blood of mice, taken 5 hours after a single intravenous injection of 2 mgm., and with the blood of normal mice for comparison revealed no difference. In a more extensive study mice were given four intravenous injections of 2 mg. on alternate days and bled one day after

TABLE 4

Influence of size of inoculum on effect of myochrysine in vitro

DILUTION OF 24-HOUR TYPE B CULTURE USED AS INOCULUM	GROWTH IN 10 PER CENT BOVINE SERUM BROTH		
	No myochrysine	Myochrysine—2 mgm. per cent (1:50,000)	Myochrysine—20 mgm. per cent (1:5000)
Undiluted	+	+	+
10^{-1}	+	+	+
10^{-2}	+	+	+
10^{-3}	+	+	+
10^{-4}	+	+	+
10^{-5}	+	+	+
10^{-6}	+	+	0
10^{-7}	0	+	0
10^{-8}	0	0	0
10^{-9}	0	0	0

the last injection. The following test was performed: 0.45 ml. of blood of myochrysine-treated mice, or of blood of normal mice, or of heparinized bovine serum broth were mixed with 0.05 ml. amounts of varying dilutions of culture, ranging from undiluted through tenfold dilutions to 10^{-6}. After 2 hours' incubation at 37°C., 0.1 ml. of each mixture was spread on a bovine serum agar plate and 0.4 ml. was transferred to tubes of bovine serum broth. Since the disintegration of blood cells after a few days' incubation gives rise to a turbidity which prevents direct reading of cultures, the presence or absence of growth was determined by subculture on the days when the control tubes without

blood showed distinct growth. By these methods the blood of myochrysine treated mice showed no antimicrobial properties *in vitro*.

(d) *Effect of growing the microörganisms in a medium containing myochrysine on their capacity to produce arthritis.* The possibility that while myochrysine may not kill or inhibit the growth of these microörganisms on cell-free media, it might perhaps abolish their capacity to produce arthritis was investigated in the following manner: (a) The microörganisms were grown for 48 hours in 25 ml. of 10 per cent bovine serum glucose broth to which 5 mgm. of myochrysine were added; a similar culture without myochrysine was set up simultaneously. Both cultures were spun on the Swedish angle centrifuge for one hour at 4,000 r.p.m., the supernatant liquid discarded, and the sediment resuspended in 10 to 12 ml. of bovine serum broth. One-half milliliter of each culture was injected intravenously into each of 10 mice; 6 of the 10 mice inoculated with the myochrysine culture and 8 of the 10 with the normal culture developed arthritis. (b) The same experiment was repeated with the modification that the microörganisms were passaged in myochrysine-containing medium for 3 generations at 48-hour intervals. Ten mice inoculated with the resuspended microörganisms of the 3rd generation myochrysine culture, and 10 mice inoculated with "normal" resuspended culture, all developed equally severe, progressive arthritis. It is clear, therefore, that *in vitro* myochrysine (in a concentration of 20 mgm. per cent) not only does not inhibit growth in cell-free media but also does not modify the ability of these microörganisms to produce arthritis.

Thus, it was not possible to demonstrate any direct antimicrobial action *in vitro* for a compound which exerts a striking curative effect *in vivo*. Any supposed stimulation of the phagocytic mechanism which is sometimes attributed to gold compounds can hardly be considered as playing a part in the curative action of myochrysine on the experimental arthritis investigated here. For it should be recalled that normal mice, by phagocytosis or otherwise, can sterilize both the blood stream and the viscera after the intravenous injection of large amounts of this microör-

ganism, and existing evidence suggests that the arthritis is a result of their special affinity for, and intracellular invasion of, mesenchymal cells of the joints. Since a distinct curative effect can be obtained even when treatment is begun several weeks after the arthritis is well established, it would appear that the beneficial action of the chemical is exerted at a time when the etiological agent is already presumably established intracellularly. It is interesting to note, therefore, that a potent immune serum or vaccine administered at the very onset of arthritis and subsequently, did not possess the curative properties of the effective gold compounds, although injection of the immune serum just before infection could prevent the development of arthritis in practically all mice (Sabin and Morgan; 1940). Whether absorption of the chemical by the parasitized cells renders them unsuitable as hosts for the infecting microörganisms or the therapeutically effective compounds modify the pathological response to the infectious process, must still be investigated.

DISCUSSION

The experimental disease utilized in the present studies differs from other types of experimental arthritis, especially those caused by pyogenic microörganisms (whether bacterial or of the rat pleuropneumonia group) in that it is a progressive, chronic, essentially proliferative arthritis resembling human rheumatoid arthritis in its clinical and pathological manifestations more than any other experimental disease produced hitherto. The fact that this experimental arthritis in mice reacts to certain gold compounds in a manner that may be considered similar to the human disease, if the existing observations in man find further confirmation, provides on the one hand an experimental basis for human therapy, and on the other suggests enough basic similarity between the two conditions to warrant the use of the experimental disease for further exploration in the field of therapy. The following data obtained in the present study would appear to have a bearing on the use of gold compounds in human therapy:

(1) The gold compounds exerting a curative effect may be

inorganic, or organic and soluble or insoluble, but in the colloidal state gold had no therapeutic value.

(2) The greatest and most rapid response was obtained with the largest doses of the soluble compounds. The same total dose administered over a period of a week gave better results than when it was given in individual smaller doses over a longer period of time. For example, 2.5 mgm. of the double thiosulfate of gold and sodium administered on alternate days in 5 doses of 0.5 mgm. each, brought about an excellent therapeutic response, while the same amount of compound given in 0.25 mgm. doses over a period of 3 weeks had little or no therapeutic effect. The present practice in human therapy of administering gold compounds at weekly intervals may actually prevent or diminish optimum effect.

(3) With borderline doses of therapeutically effective compounds there is a high incidence of recurrence after the cessation of treatment.

(4) The earlier in the disease treatment was begun, the more rapid and complete was the therapeutic response. When treatment was delayed for as long as 4 weeks (this coincides approximately with the stage when distinct cartilage destruction is first observed in the mouse arthritis) the involvement of some joints, although not all, had progressed to a point where therapy was no longer effective. The necessity of evaluating this therapy in early cases of human rheumatoid arthritis is thus emphasized.

(5) Tests with calcium aurothioglycolate, an insoluble organic gold compound, indicated that while it is tolerated in very large doses, enough active substance is absorbed over a period of weeks to bring about a complete and persistent curative effect. The desirability of investigating the obvious applications of such a compound in the human disease, which progresses very much more slowly than the mouse arthritis, is self-evident.

(6) Toxicity and therapeutic effectiveness of the gold compounds, studied thus far, are not parallel. Thus, the double thiosulfate of gold and sodium (sanocrysin) had a chemotherapeutic index of approximately 2 for the intravenous route, while

under identical conditions the index for sodium aurothiomalate (myochrysine) was 30.

All chemotherapeutic agents, including the arsphenamine and sulfonamide compounds, have a certain degree of toxicity and danger associated with their use, and, therefore, the probable benefits to be derived must always be weighed against the possible dangers involved. Whether the incidence of serious toxic reactions with suitable gold compounds is greater than with certain other chemotherapeutic agents now used in human therapy is problematical. However, while among the compounds we have tested thus far there is already one, sodium aurothiomalate (myochrysine) with a chemotherapeutic index of approximately 30, it is not unlikely that other gold compounds exist or may be synthesized with a still greater margin of safety. This assumption is based on the demonstration in the present study that different factors are involved in determining the minimal lethal dose and the minimal therapeutic dose of a compound. Further work, therefore, will concern itself not only with a systematic study of the relationship between chemical structure and the chemotherapeutic index of existing and newly synthesized gold compounds, but also with a search for other chemical agents with similar curative properties.

SUMMARY

1. Certain inorganic and organic gold compounds, of both the aliphatic and aromatic series, have been found to exert a curative effect on an experimental, proliferative, chronic arthritis in mice produced by a filtrable microörganism of the pleuropneumonia group which is a normal inhabitant of the upper respiratory tract of mice.

2. While colloidal gold compounds were ineffective, tests with an "insoluble," organic compound (calcium aurothioglycolate) revealed that enough active substance is absorbed over a period of weeks to bring about a complete and persistent curative effect.

3. The greater the dosage of an effective compound the more rapid and complete is the disappearance of the arthritis. A given total dosage which is curative when administered in a small

number of divided doses within a short period, may be ineffective when it is divided in a large number of small doses and given over a much longer period of time.

4. With doses on the borderline of effectiveness there is a high incidence of spontaneous recurrence after cessation of treatment.

5. The earlier in the disease treatment is begun, the more rapid and complete is the therapeutic response. When treatment was delayed for 4 weeks after the onset of arthritis, the involvement of some joints in the majority of mice had progressed to a point where therapy was no longer effective.

6. Arthritis disappeared completely in 96 per cent of 171 mice which were treated with suitable gold compounds in adequate dosage and at the proper time, but only in 5 per cent of 77 mice which were treated with other compounds such as colloidal gold, bismuth subsalicylate, sodium salicylate, neoarsphenamine or sodium sulfapyridine, and in only 7 per cent of 70 mice which remained untreated.

7. Neither the therapeutically effective compound *per se*, nor the blood of mice treated with it in large doses prevented the growth of the etiological agent *in vitro*; furthermore, microörganisms grown in the presence of an active gold compound for 3 generations did not lose their capacity to produce arthritis.

8. Toxicity and therapeutic effectiveness were found to depend upon different properties of the same compound. Thus, the margin of safety as represented by the chemotherapeutic index $\left(\dfrac{\text{minimal lethal dose}}{\text{minimal curative dose}}\right)$ varied from approximately 2 for the thiosulfate of gold sodium (sanocrysin) to about 30 for sodium aurothiomalate (myochrysine).

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PSEUDO-COLONIES SIMULATING THOSE OF PLEURO-PNEUMONIA-LIKE MICROÖRGANISMS

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While attempting to cultivate filterable pleuropneumonia-like microörganisms from patients with rheumatic fever, we encountered pseudo-colonies, which resembled those previously described by Twort and Twort (1921) and Laidlaw (1925); and some additional information concerning these artifacts was obtained. Because of the current interest in pleuropneumonia-like microörganisms, it is felt that the confusion that might ensue from the appearance of these pseudo-colonies should be more widely appreciated.

METHODS

The media in which these pseudo-colonies were first seen consisted of 30 per cent horse serum agar. The serum was obtained from the New York Board of Health Laboratories, and was filtered under positive pressure of 20 lbs. through Seitz pads, grade EK. Freshly filtered serum was heated to 45°C. and mixed with freshly melted nutrient agar. About 20 ml. of the mixture were poured into Petri dishes having a piece of sterile filter paper under the cover. The dishes were sealed with parafilm, and incubated for 24 to 48 hours to insure sterility, then stored on a laboratory table. After being inoculated with suspected material, the Petri dishes were again sealed with parafilm and incubated at 37°C. Thus, during both the periods of storage and incubation, the filter paper prevented moisture from running over the surface of the medium; and the tight seal of parafilm insured a continuous moist atmosphere about the media. These cultural conditions are very favorable for growing several strains

of pleuropneumonia-like microörganisms; in fact, Dr. Albert Sabin (1938) has found them the most satisfactory for growing easily on solid medium those strains carried by many mice.

Cultures prepared in this way have an additional advantage: they may be repeatedly examined microscopically without being opened and exposed to air contaminants. The Petri dish is placed cover side down on an ordinary microscopic stage; and the filter paper in the cover is illuminated with a very fine beam of light obtained by almost completely closing the iris diaphragm under the condenser and sharply focusing this beam on the paper. By manipulating the mirror, and thus moving the beam of light, either flat or oblique illumination may be provided, and the contour of the colonies studied. Both the culture medium and the Petri dishes must be crystal clear. For preliminary examination, a low power, long focal distance planar objective has been found convenient, and for more detailed study the usual low power objective ($10 \times$) with a $10 \times$ ocular. A particular zone on the medium may be marked by sticking over this zone a piece of finely perforated paper. Scotch adhesive tape has proven a useful fastener for this paper. By numbering each bit of paper, the areas can be easily identified, and the underlying colonies can be repeatedly found and studied or used for subcultures.

Appearance of pseudo-colonies

On horse-serum agar a characteristic pseudo-colony begins as a highly refractile globule having a slightly yellowish tinge, the so-called "amber body"; around this there develops an irregular circular zone finely granular in texture and often with a radially arranged wrinkled appearance at the periphery (fig. 1). Oblique illumination gives the impression of a slight excavation of the medium, or the appearance of a very flat crater containing at its center the brilliant "amber body." This depression of the surface is especially noticeable when contrasted with the raised contour of a true pleuropneumonia-like colony (fig. 2). Under the cultural conditions described, these pseudo-colonies develop much more slowly than do those of most pleuropneumonia-like microörganisms; the "amber bodies" may not appear for 4 to 7

days after incubation at 37°C.; and the fully developed pseudo-colony may not attain its maximum size of from 50 to 150 μ for 10 to 15 days. Like true colonies, these present their characteristic morphology only when widely separated; when they are closely crowded, their macroscopic appearance on the surface of the medium is that of a film-like growth, and the microscopic picture that of numerous "amber bodies" with slight or no surrounding pseudo-colony formation (fig. 3). In old uninoculated plates, minute refractile bodies of various sizes form in the deeper parts of the medium; these sometimes resemble spores of molds, or appear like concave discs, and occasionally look simply like the globular "amber bodies." As noted by Laidlaw, similar microscopic globules develop in serum or serum broth that has been incubated several days, and when numerous, they form a fine sand-like sediment which may be seen macroscopically, or better with a hand lens, and which rises in the medium in the form of a fine whorl when the tube of liquid is shaken.

While the pseudo colonies have developed most vigorously on agar containing horse serum, they were also found on media made with rabbit, human and beef serum. The serum from each species gave pseudo-colonies with certain peculiarities. On rabbit serum agar the periphery of the pseudo-colony was more distinctly outlined and the surface more clear cut as though etched with a sharp tool; an "amber body" was often absent or small and excentrally placed (fig. 4). In the media surrounding the larger pseudo-colonies, the globules were usually less numerous and more uniform in size than those appearing in horse serum agar (fig. 4, fig. 5). On media made with ascitic fluid or human serum, the pseudo-colony appeared as a small concave disc (fig. 6), with no visible change in the surrounding medium; and in the depths of the agar relatively few refractile globules appeared. These pseudo-colonies were often distributed in streaks throughout the media and not uniformly as in solid media made with other sera. On beef serum agar there first appeared an "amber body" or concave disc, on one side of which there were subsequently formed curved or flat S-shaped lines radially ar-

ranged, so that the appearance was that of a cocks-comb (fig. 7). Subsequently, similar lines appeared about the entire pseudo-colony and gave it the appearance of a pin-wheel with a fairly large, highly refractile center. Relatively few round globules were seen in the deeper part of the agar.

While the forms described above were seen on uninoculated media that had been incubated at 37°C. for 10 to 14 days, or on plates that had stood at room temperature from 2 to 4 weeks, they appeared more readily on media inoculated with agar containing a few pseudo-colonies that had arisen spontaneously. For example, if a small block of agar containing one or two pseudo-colonies was cut out with a sterile spud and rubbed over the surface of a fresh plate, many pseudo-colonies developed along the line of inoculation, with characteristic morphology at the margins of the inoculated zone or where the pseudo-colonies were widely separated, and small, poorly defined pseudo-colonies in the middle of the inoculated areas. If this crowded zone were cut out and used as an inoculum, the subculture consisted almost entirely of the nontypical crowded forms. In this respect, as noted also by Laidlaw, the phenomenon of multiplication resembles that of microörganisms. After inoculating a serum agar plate with minced tissue or exudate, the pseudo-colonies usually developed more numerously along the inoculated streaks, a phenomenon which might easily mislead the observer into thinking that they had been derived from the inoculum. Moreover, simply disturbing the surface of the medium by rubbing with a spud, or by "inoculating" it with sterile salt solution or broth, sometimes similarly caused the pseudo-colonies to appear and "grow" most vigorously along the "inoculated" areas; hence it seems that the favoring factor may be mechanical rather than viable. When four blocks of horse serum agar, having on their surface approximately the same amount of "growth" were inoculated on the surface of fresh horse serum, rabbit serum, ascitic fluid, and beef serum agar, respectively, "growth" first appeared and was most vigorous on the horse serum agar subculture; it was somewhat slower in appearing and less vigorous on the rabbit serum agar; and only relatively few pseudo-colonies appeared on

the inoculated areas of the human ascitic fluid and beef serum agar. On each medium, moreover, morphology of the well-separated pseudo-colonies was typical for the respective medium. This phenomenon of formation of pseudo-colonies imitates the tendency for certain pleuropneumonia-like microorganisms to exhibit somewhat different but characteristic colonies on media containing serum from different animal species. "Cut-outs," placed in broth containing serum from these four different species of mammals, also showed the formation of refractile globules, most abundant in the horse serum and diminishing in intensity parallel to that on the respective solid media.

When agar was mixed with diminishing concentrations of horse serum from 30 to 0.1 per cent, all media containing over 5 per cent developed numerous pseudo-colonies, those containing between 3 and 1 per cent, less numerous; while those having only 0.5 to 0.1 per cent showed none. Media made with old or freshly obtained serum were equally favorable for the development of the pseudo-colonies, as were media made with citrated serum. On the other hand, the addition of 0.2 per cent sodium oxalate to the serum, completely inhibited the appearance of these pseudo-colonies.

The effect of applying certain measures that would influence the growth of filterable microörganisms was tested. Serum, which, unfiltered, yielded a good "growth" on serum agar plates, gave approximately as vigorous "growth" when filtered through Berkefeld V and W candles, Chamberland L3, L5 and L7 filters, and through Seitz EK pads of both American and German manufacture. Horse serum filtered directly through a Gradacol membrane of APS 100 m.mu. yielded a fair number of colonies when made into serum agar plates, but fewer than control plates made with unfiltered serum. Horse serum agar plates, made and sealed in the usual way, then heated to 60°C. for one hour, yielded many typical colonies; that heated to 65°C. for one hour still developed the characteristic pseudo-colonies to about the same degree as unheated controls. Plates made with serum heated even to 70°, although containing considerable coagulum, still gave some "growth," chiefly of highly refractile

bodies.   Thirty per cent horse serum agar containing merthiolate in a concentration of 1:500, yielded pseudo-colonies like those on the control media, even though it was impossible to grow any bacteria on such chemically treated medium; and pseudo-colonies appeared on serum agar containing formalin in concentrations of 0.5, 1.0 and 5.0 per cent respectively; but in the 5.0 per cent concentration, the "growth" consisted chiefly of the finer globular bodies without the characteristic surrounding crater-like zone. The failure of these disinfecting measures to prevent the appearance of pseudo-colonies seemed to place the causation of the phenomenon outside the action of living microörganisms.

Laidlaw, by analyzing chemically the sediment consisting of highly refractile globules grown in serum, concluded that these globules consisted of calcium and magnesium soaps, and, by analogy, that the pseudo-colonies, he found on serum agar slants, were composed of the same material which, he thought, formed the spherocrystals that apparently comprise most of the pseudo-colonies.   Because direct proof of the chemical constitution of these pseudo-colonies was lacking, we attempted to stain them in various ways.   Contact films and sections of the serum agar pseudo-colonies stained with Giemsa revealed purplish globules of various sizes but with no uniform structures that could be definitely identified.   Staining the colonies directly with Scharlach R or osmic acid did not give any differential diagnostic staining; but when the agar containing pseudo-colonies was first fixed a few minutes with 4 per cent formalin, then was stained with Scharlach R and counterstained with methylene blue, the pseudo-colonies took the typical red color of fatty substances against a blue background of agar.   With this stain, pseudo-colonies on horse serum agar had a large homogenous central globule, which was probably the "amber body" seen in unstained preparations; this was surrounded by a disc-shaped structure composed of very fine red granules.   Pseudo-colonies appearing on horse serum agar containing formalin, stained characteristically red immediately following treatment with Scharlach R.   These staining reactions confirm Laidlaw's ideas concerning the fatty nature of the "growths."

DISCUSSION

The phenomenon here described is of interest from several angles. Unless recognized in their various forms, these pseudo-colonies, composed of material which takes a fat stain, may be confused with the growth of filterable microörganisms, for not only in their morphology do they mimic colonies of pleuropneumonia-like microörganisms, but by applying the cultural techniques commonly used to propagate these microörganisms in subcultures, the pseudo-colonies appear to multiply to a remarkable degree. Doubtless, in subculturing them by rubbing one or two over the surface of fresh medium, minute bits of these pseudo-colonies are distributed over the serum agar, and each bit forms a nidus specially favorable for the crystallization of new spherocrystals out of the medium. The procedure of adding small amounts of a given substance to a solution of that substance to favor and hasten its crystallization from solution is commonly employed by chemists. The peculiarity in the particular example of this general phenomenon here cited is that the resulting crystals so closely mimic the appearance of certain microörganisms, not only in their general morphology, but in the manner in which they present forms characteristic for the different sera out of which they may have crystallized. Here again chemical parallels may be cited, for the forms of crystals may be characteristically conditioned by the medium out of which they have precipitated. Finally, their spontaneous formation under suitable conditions or where the medium is physically disturbed, such as in areas inoculated with exudates or minced tissue, is comparable to the deposition of crystals from chemical solutions following the application of certain physical disturbances to that solution.

SUMMARY

Pseudo-colonies are described resembling somewhat those formed by filterable microörganisms, both in their morphology and in the manner in which they may be propagated on serum agar and in serum broth. Media made with sera from different

animal species form pseudo-colonies characteristic for that species. The pseudo-colonies are composed largely of sphero-crystals that stain typically with fat stains. These observations extend and amplify those previously made by the Tworts and by Laidlaw.

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PLATE 1

FIG. 1. Thirty per cent horse serum agar incubated 14 days. Pure "growth" on 10th subculture. Flat illumination. Central "amber bodies" very marked. ×115.

FIG. 2. Thirty per cent horse serum agar. Inoculated with minced pneumonic mouse lung, incubated 7 days. Oblique illumination. Two smaller crater-like pseudo-colonies and one larger colony of pleuropneumonia-like microorganisms, Type A Sabin. ×115.

FIG. 3. Thirty per cent horse serum agar, heavily "inoculated," incubated 12 days. Crowded pseudo-colonies with numerous "amber bodies" and little peripheral "growth." ×115.
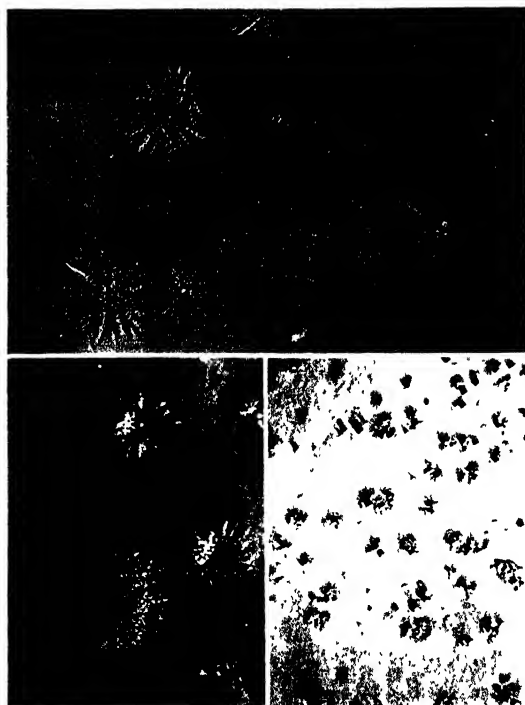
(T. M. Brown, H. F. Swift and R. F. Watson. Pseudo-colonies on Serum Agar)

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PLATE 2

FIG. 4. Pseudo-colonies in 30 per cent rabbit serum agar, incubated 2 weeks ×115.

FIG. 5. Thirty per cent rabbit serum agar, incubated 6 weeks. Pseudo-colonies have marked chiseled appearance.   ×115.

FIG. 6. Thirty per cent ascitic fluid agar, incubated 2 weeks   Pseudo-colonies in form of small discs with central depression.   ×115.

FIG. 7. Thirty per cent beef serum agar, incubated 1 week.   Small central concave disc with curved peripheral areas forming cocks-comb pseudo-colonies ×115.
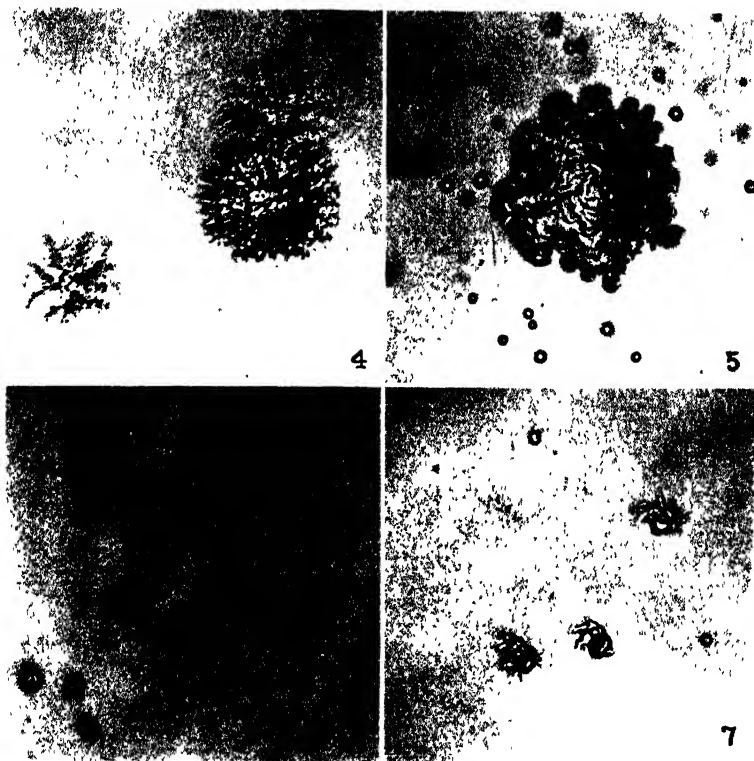
(T. M. Brown, H. F. Swift and R. F. Watson Pseudo-colonies on Serum Agar)

PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

KENTUCKY BRANCH

SECOND MEETING, UNIVERSITY OF KENTUCKY, LEXINGTON, OCTOBER 25, 1940

PRELIMINARY STUDIES ON PINK MARGARINE. *William E. Johnston and Ernest E. Pittman*, State Board of Health, Louisville, Kentucky.

NEW TYPES WITHIN GROUP C OF THE GENUS SALMONELLA. *P. R. Edwards and D. W. Bruner*, Department of Animal Pathology, Experiment Station, University of Kentucky, Lexington, Kentucky.

A COMPARISON OF THE VIRUSES OF STREAK OF TOBACCO AND YELLOW DWARF OF POTATO. *W. D. Valleau*, Agronomy Department, Experiment Station, University of Kentucky, Lexington, Kentucky.

A comparison of host range and physical characters of tobacco-streak virus and potato-yellow-dwarf virus indicates that they are similar, if not strains of the same virus. Tomato and potato, previously thought immune, were found to be hosts of the tobacco streak virus. The potato-yellow-dwarf virus is known to affect red clover and circumstantial evidence indicates that the streak virus is carried to tobacco from sweet clover. Symptoms of yellow dwarf and streak are not identical either in *Nicotiana rustica* or in *N. tabacum*. In the latter, yellow-dwarf causes vein clearing and no streak. Tobacco with systemic yellow-dwarf developed a combination disease when inoculated by grafting

with the streak virus. The fact that the yellow-dwarf virus does not protect against the streak virus is not necessarily considered proof that the viruses are of different kinds. They may be necrotic and non-necrotic strains of the same virus.

THE EFFECT OF FERMENTATION ON THE THIAMINE AND RIBOFLAVIN CONTENT OF GRAIN MASHES. *G. A. Ratti, Jr., W. H. Stark, and Paul Kolachov*, Research Department, Microbiology Division, Joseph E. Seagram and Sons, Inc., Louisville, Kentucky.

Fermentation balances for riboflavin and thiamine were run on laboratory and plant fermentations of corn spirits and 35% small grain-bourbon mashes. The samples were assayed for riboflavin by the method of Snell and Strong. Thiamine was determined by the ultramicro method of Atkin, Schultz, and Frey. Corn spirits mash was found to contain 0.59 gamma of riboflavin per ml. which increased to 0.78 gamma during 72 hour fermentation, an increase of 32.2%. The individual components of the corn spirits mash were assayed for riboflavin and the calculated content (0.57 gamma per ml.) and the actual assayed content (0.54 gamma per ml.) showed close agreement. These values are for the uninoculated mash. Plant bourbon mash increased in riboflavin content from 0.46 to 0.65 gamma per ml. (41.3%)

during fermentation, while the thiamine content rose from 0.80 to 0.98 gamma per ml. (22.5%).

CLEANING AND DISINFECTION OF THE WOODEN GRIDS IN A LOW TEMPERATURE SPIRIT STILL. *S. L. Adams, H. R. Bilford, and W. H. Stark*, Research Department, Microbiology Division, Joseph E. Seagram and Sons, Inc., Louisville, Kentucky.

In order to operate the beer column of a new spirits still at a temperature of 95°F., it was necessary to substitute wooden grids for the usual plates. The operating conditions were anaerobic. The problem was to devise a method of cleaning and disinfecting the grids.

A 0.5% solution of sodium hydroxide was found to be a satisfactory cleaning agent. The disinfecting action of copper sulphate, sodium fluoride, hydrofluoric acid (35.5%), H.T.H. (calcium hypochlorite), and Montanin (hydrofluosilicic acid 32%) was tested.

Copper sulphate and sodium fluoride proved ineffective even in concentrations of 10%. H.T.H., though not tested fully because of the possibility of its imparting an odor to the distillate, was ineffective in a 0.1% solution. 2% to 5% solutions of hydrofluoric acid inhibited growth for 24 hours, but the dangerous nature of the acid was objectionable. Treatment for 15 minutes with a 5.0% solution of Montanin preceded by a 0.5% caustic wash inhibited bacteriological growth for 24 hours. Chips given this treatment had no off odor after incubation in a moist chamber.

This last procedure is now being used in the plant with satisfactory results. The concentration of caustic has been increased to 1.0% and the time of exposure increased to 45 minutes for plant use.

SOCIETY OF ILLINOIS BACTERIOLOGISTS

CHICAGO WOMAN'S CLUB, CHICAGO, ILLINOIS, OCTOBER 18, 1940

PREPARATION AND STERILIZATION OF SURGICAL CATGUT. *J. H. Glynn*, Armour Laboratories.

BACTERIOGENIC HEMAGGLUTININS. *Israel Davidsohn and B. Toharsky*, Mt. Sinai Hospital.

ISOLATION OF TYPHOID BACILLI FROM DRINKING WATER. *H. J. Shaughnessy*, Chicago Branch, Illinois State Department of Health.

PRODUCTION OF STAPHYLOCOCCUS ENTEROTOXIN IN CANNED CORN, SALMON AND OYSTERS. *Ellen Davison*, Department of Bacteriology, University of Chicago.

Cans of corn, salmon and oysters were inoculated with a strain of enterotoxic staphylococci, incubated for 3 to 7 days and the contents assayed for enterotoxin by feeding of human volunteers and by intravenous injection of concentrates or filtrates into monkeys. Concentration of enterotoxin from foods by precipitation with ammonium sulfate cannot be recommended for routine assay. With experimentally inoculated food under conditions most favorable for the growth of staphylococci, we were able to demonstrate enterotoxin in only a small per centage of cases. Intravenous injection of the material, which is a more delicate test than feeding, must be carefully controlled to rule out non-

specific reactions and individual idio-syncracies. Certain staphylococci can elaborate an enterotoxin in canned corn and in canned oysters. This was demonstrated by intravenous injection of monkeys and feeding of human volunteers. We were unable to demonstrate staphylococcus enterotoxin in inoculated canned salmon. The salmon was assayed by intravenous injection into monkeys and by feeding of human volunteers. Concentration of enterotoxin from foods by precipitation from saturated salt solution can be accomplished, but does not give a high enough percentage of positive results to be used as a practical routine method of assay.

INDEX TO VOLUME 40